IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Janet L. Andres, Ph.D.
Art Unit	:	1646
Applicants	:	Howard M. Johnson, Mustafa G. Mujtaba
Serial No.	:	09/648,864
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Conf. No.	:	6790
For	:	Materials and Methods for Inhibition of IgE Production

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF HOWARD M. JOHNSON, Ph.D. UNDER 37 CFR §1.132

Sir:

I, Howard M. Johnson, Ph.D., hereby declare:

THAT, I am a co-inventor of the subject matter claimed in U.S. patent application Serial No. 09/648,864 (hereinafter the '864 application);

THAT, I have read and understood the '864 application;

THAT, I have read and understood the rejection of claims in the Office Actions mailed January 28, 2002, October 21, 2002, and May 19, 2003 in the '864 application;

AND, being thus duly qualified, do further declare:

The claims of the subject application are rejected under 35 USC §103(a) as obvious over the publications by Pene *et al.* (1988), Gruschwitz *et al.* (1993), or Kimata *et al.* (1995), and further in view of Johnson *et al.* (WO 97/39127). The Examiner asserts that the cited primary references teach the use of interferon alpha to downregulate IgE production. While acknowledging that the primary references do not teach that interferon tau can downregulate IgE production, the Examiner asserts that it would have been obvious to substitute interferon tau for interferon alpha in view of the Johnson *et al.* reference which, according to the Examiner, teaches that interferon alpha and interferon tau bind to the type I receptor and have similar

biological activities. From this, the Examiner concludes that the ordinarily skilled artisan, at the time of the subject invention, would have expected that interferon tau, like interferon alpha, would also downregulate IgE production.

I respectfully assert that type I interferons do not share all of the same biological activities and that an ordinarily skilled artisan, at the time of our invention, would not have reasonably expected that interferon tau would downregulate IgE production. One of the primary distinctions between interferon tau and other type I interferons is that there is a significant difference in the toxicity associated with interferon tau versus other interferons. Interferon tau is substantially less toxic to eukaryotic cells than interferon alpha. In my opinion, this is a qualitative difference between interferon tau and the other interferons.

There are other qualitative differences between interferon tau and other type I interferons, such as apoptosis inducing activity. In my laboratory, interferon tau was compared with human interferon alpha 2A for induction of apoptosis on the human Daudi cell line (Subramaniam *et al.*, 1998, "Type I interferon induction of the Cdk-inhibitor $p21^{WAF1}$ is accompanied by ordered G1 arrest, differentiation and apoptosis of the Daudi B-cell line" *Oncogene*, Vol. 16, pp. 1885-1890). As shown in Figure 4 of the Subramaniam *et al.* publication, Daudi cells treated with interferon tau exhibited normal morphology, similar to that of untreated control cells. In contrast, exposure of Daudi cells to interferon alpha 2A, at 10-fold less concentration, produced classic morphologic changes characteristic of cellular apoptosis (McConkey *et al.*, 1996, "Signal Transduction Pathways in Apoptosis" *Stem Cells*, Vol. 14, pp. 619-631). These changes consist of membrane blebing and nuclear fragmentation that is indicative of chromosome disruption. These results show that interferon tau does not induce cellular apoptosis under conditions where interferon alpha does induce apoptosis in a human cell line. The above described apoptosis inducing activity is an example of another qualitative, functional distinction between interferon tau and other type I interferons.

In a recent study, it was shown that interferon tau promotes a T-helper 2 (Th2) bias in suppression of autoimmune encephalomyelitis in a mouse model of multiple sclerosis (MS) (Soos *et al.*, 2002). Specifically, it was shown that oral administration of interferons alpha, beta and tau to myelin basic protein-specific T-cell receptor transgenic mice promoted secretion of

the Th2 cytokine interleukin-10 with similar efficiency. Unexpectedly, however, interferons alpha and beta also induced secretion of the T-helper 1 (Th1) cytokine interferon gamma, whereas interferon tau did not induce secretion of interferon gamma. Activation of Th1 cells, with the subsequent production of interferon gamma, can result in proinflammatory responses, which may explain some of the undesirable side effects of interferon beta treatment of multiple sclerosis. Interferon tau does not activate the Th1 arm of the T-cell system. These differences between interferons alpha, beta and tau could not have been predicted based on simple reference to receptor specificity.

Similar to interferon gamma, type I interferons have been shown to possess STAT-independent functions (Gongora *et al.*, 2000). Specifically, it has been shown that inhibition of interleukin-7-dependent B lymphopoiesis by interferon alpha or beta is unaffected in STAT-1-deficient mice. The data indicate that type I interferons can activate an alternative signaling pathway in which STAT1 is not an essential component. Although interferons are classified into groups or types, they possess aspects of unique structure such as their differences in amino acid composition. These differences could play a role in the non-STAT transcription factor function of these individual type I interferons. In particular, interferon tau contains more amino acid residues than do alpha interferons, as well as more differences in the sequence when compared to alpha interferons as a group (Bazer and Johnson, 1991). Even among alpha interferons, there are unpredictable differences in function. It has been shown recently, for example, that five subtypes of human alpha interferons exhibited different anticellular activity against chronic myelogenous leukemia cells (Yanai *et al.*, 2002). The authors state that "these data indicate in vitro distinctions between IFN-α subtypes that should be appreciated more in the clinic." (emphasis added)

Thus, while interferons tau and alpha (and other type I interferons) may have some similar biological activities, they also exhibit significant differences in activities. Accordingly, I respectfully submit that an ordinarily skilled artisan, at the time of our invention, could have only speculated that interferon tau would downregulate IgE production.

Accordingly, I respectfully maintain, based on my numerous years of involvement in interferon research and on the evidence presented herein, that the invention claimed in the '864 application is not obvious over the cited references.

A copy of each publication cited herein is attached to this Declaration.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: Howard M. Johnson

Howard M. Johnson, Ph.D.

Date: August 29, 2003

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SHORT REPORT

Type I interferon induction of the Cdk-inhibitor p21^{WAF1} is accompanied by ordered G1 arrest, differentiation and apoptosis of the Daudi B-cell line

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We show, in this study, that type I IFN induction of the cyclin-dependent kinase (cdk) inhibitor p21^{WAF1} in the human Burkitt lymphoma B cell-line Daudi and ensuing cell cycle arrest correlate with the terminal differentiation of these cells, and is ultimately followed by apoptosis and cell death. The expression of p21^{WAF1} paralleled the onset of G1 arrest and the reduction of surface IgM expression which was used as a marker of the differentiation response, and the IFN treated cells acquired a typical plasma cell-like morphology. The type II IFN IFNγ, which does not inhibit the growth of Daudi cells, did not induce the expression of p21^{WAF1}, nor affect the expression of surface IgM. The induction of p21^{WAF1} which paralleled the inhibition of the phosphorylation of the retinoblastoma protein, pRB, was preceded by the strong reduction in c-*myc* levels. We propose that the coupled down-regulation of c-*myc* and induction of p21^{WAF1} may be crucial to the induction of differentiation and G1 arrest in Daudi cells by type I IFN. Growth arrest and differentiation was followed by apoptosis and cell death, and was accompanied by the induction of the activity of the apoptotic ICE-family protease CPP32. G1 arrest and differentiation followed by apoptotic cell death are characteristics of terminal differentiation. Thus, our data suggest that the induction of p21^{WAF1} and G1 arrest mediated by type I IFN in Daudi cells is part of terminal differentiation response in these cells, highlighting a role for type I IFN as B cell terminal differentiation factors.

Keywords: interferon α; p21^{WAF1}; B-cell differentiation; apoptosis; cell cycle

Members of the type I interferon family (IFN) that include IFNα, IFNβ, IFNω and IFNτ are strong antiproliferative agents that can induce G1 arrest in many cell types (Tamm *et al.*, 1987). The Burkitt lymphoma B cell-line Daudi is particularly sensitive to the antiproliferative effects of the type I IFN and has been routinely used in exploring the mechanisms of the G1 arrest induced by type I IFN.

In Daudi cells, IFN-induced G1 arrest is accompanied by several changes in proteins associated with the cell cycle that are interrelated at the molecular level. These events include reduction in expression of c-*myc* (Einat *et al.*, 1985) and cyclin D3 (Tiefenbrun, *et al.*, 1996) hypophosphorylation of pRB (Thomas *et al.*, 1991; Burke *et al.*, 1992; Kumar and Atlas, 1992; Resnitzky *et al.*, 1992; Zhang and Kumar, 1994), suppression of E2F DNA-binding activity (Melamed *et al.*, 1993) and inhibition of the activity of the cyclin-dependent kinase (cdk) cdk2 (Zhang and Kumar, 1994; Tiefenbrun *et al.*, 1996) associated with the cyclins cyclin E and cyclin A. The expression of the tyrosine phosphatase cdc25A has also been reported to be inhibited (Tiefenbrun *et al.*, 1996).

We and others have previously shown that another key cell-cycle regulatory molecule, the cdk-inhibitor p21^{WAF1}, is induced following IFN treatment of Daudi cells (Subramaniam and Johnson, 1997; Sangfelt *et al.*, 1997), and in Daudi cells increased levels of p21^{WAF1} can be found complexed with cdk2 following IFN treatment. This is accompanied by inhibition of cdk2 activity and G1 arrest.

Besides its role in regulating the cell cycle, the cdk-inhibitor p21^{WAF1} appears to play an important role in the differentiation of many cell types (reviewed in Gartel *et al.*, 1996). Cellular differentiation is preceded by G1 arrest and exit from the cell cycle. The treatment of Daudi cells with type I IFN induces many phenotypical changes characteristic of the terminal differentiation of B cells to plasma cells (Exley *et al.*, 1987a,b; Clemens *et al.*, 1988). Thus, in this study, we have further investigated the temporal relationships between p21^{WAF1} expression, molecular changes during G1 arrest and the differentiation of Daudi cells following IFN treatment.

The treatment of Daudi cells with IFNα leads to a rapid induction of the protein levels of p21^{WAF1} that was evident at 6–8 h of treatment and increased over a 20 h period (Figure 1a). The induction of p21^{WAF1} paralleled the accumulation of cells in the G1 phase of the cell cycle (Figure 1a: % of cells with 2N DNA content). Thus, there is a close link between the induction of p21^{WAF1} and G1 arrest induced by IFNα. In contrast, the type II IFN, IFNγ, had no effect on the levels of p21^{WAF1} in Daudi cells (Figure 1a) which is consistent with the fact that IFNγ does not inhibit the growth of Daudi cells (Tomita *et al.*, 1982; Rubin *et al.*, 1983; data not shown). This lack of effect of IFNγ is specific to Daudi cells since in DU145 cells, a human prostate cancer cell line, IFNγ treatment clearly induced the expression of p21^{WAF1} (Figure 1b) and also inhibited the progression of DU145 cells through the G1 phase of the cell cycle (Hobeika, *et al.*, manuscript submitted). Thus, type I IFN induction of the cdk-inhibitor p21^{WAF1} correlates with the inhibition of the cell cycle of Daudi cells in G1.

We next determined the temporal relationship of the induction of p21^{WAF1} by type I IFN to changes in other cell cycle-associated proteins. As can be seen from

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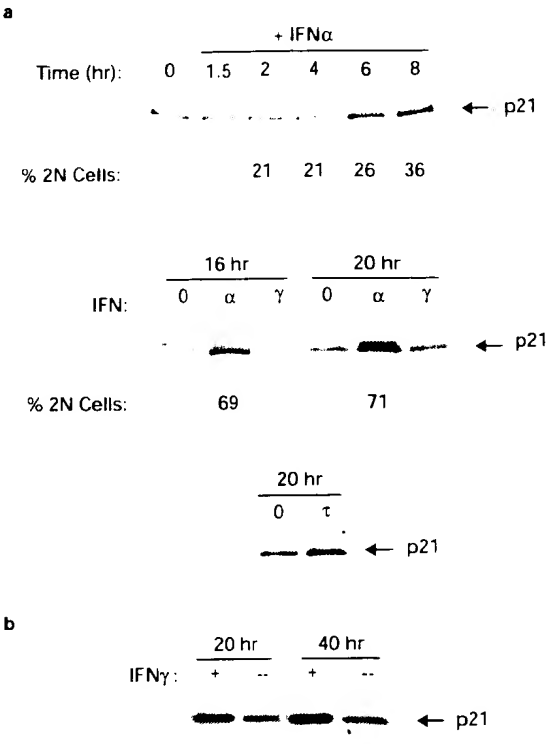

Figure 1 Induction of p21[WAF1] by IFNα in Daudi cells (**a**). Daudi cells at approximately 2×10^5 cells per ml were treated in complete growth medium (RPMI-1640 supplemented with 20% fetal bovine serum) with IFNα (320 U/ml), or IFNγ (400 U/ml) for the indicated time periods and p21[WAF1] expression assayed by immunoprecipitation and immunoblotting from cell extracts as described earlier (Subramaniam and Johnson, 1997). For cell cycle analysis cell samples containing approximately 2×10^6 cells per ml were prepared and stained with propidium iodide using previously described procedures (Noguchi, 1992). Data from 50 000 recorded events were used for cell cycle analysis using the CellFIT program (Subramaniam and Johnson, 1997). (**b**). Effect of IFNγ treatment of DU145 cells (at 2×10^5 cells per ml) on the expression of p21[WAF1]. Total cell extracts from treated and untreated cells were analysed by SDS–PAGE and immunoblotting. For the detection of proteins from total cell extracts, cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 mM β-glyceroyl phosphate, 2 mM Na_3VO_4, 10–20 μg/ml each of leupeptin, pepstatin and aprotinin, 5 μg/ml of benzamidine, 50 μM p-nitrophenyl guanadinobenzoate, 1% Triton-X 100, 0.25% Na-deoxycholate, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride. Approximately 80–100 μg total protein from extracts was used for subsequent SDS–PAGE and immunoblotting. α, IFNα; γ, IFNγ; τ, IFNτ

Figure 2, IFNα treatment caused a rapid reduction in the expression of c-*myc* and cyclin D3 proteins (Figure 2a and b). These events were accompanied by reduced phosphorylation of pRB, as evidenced by the increase in migration of the hypophosphorylated forms by 8 h following treatment. However, the levels of the cell cycle tyrosine phosphatase cdc25A, which activates cdk2, were not affected by IFN treatment. The changes in cyclin D3 and pRB appear to occur within a similar time frame as the induction of p21[WAF1] and are consistent temporally with the onset of type I IFN-induced G1 arrest in Daudi cells. The reduction of c-*myc* levels appears to be the earliest event, but is closely followed by the induction of p21[WAF1]. This

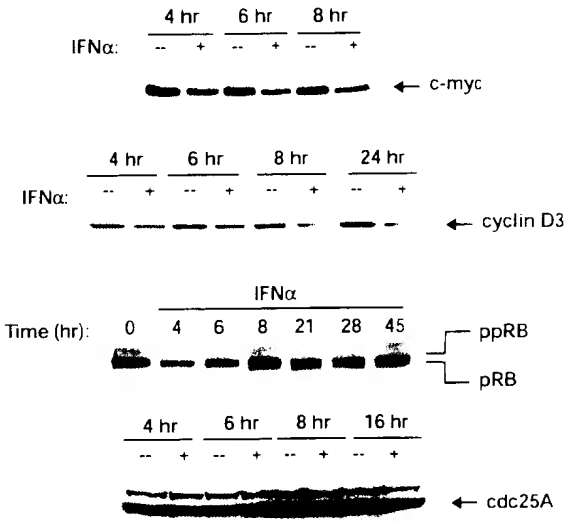

Figure 2 Effect of IFNα (320 U/ml) on the expression of various cell-cycle associated proteins in Daudi cells. (**a**) Equal amounts of protein, within a given experiment, from total cell extracts prepared as described in Figure 1b from untreated and IFN treated cells were subjected to SDS–PAGE and immunoblotting and membranes probed with antibodies to c-*myc*, pRB and cdc25A. The c-*myc* blot was stripped and reprobed for cyclin D3

reciprocal down-regulation of c-*myc* and induction of p21[WAF1] and growth arrest, shown here in IFN-treated Daudi cells, is analogous to that seen in other haematopoeitic cells where c-*myc* down regulation also appears to be tightly coupled to p21[WAF1] induction and growth arrest (Steinman *et al*., 1994; Blagosklonny *et al*.., 1996). Moreover, this inverse relationship between c-*myc* and p21[WAF1] is also linked to the differentiation of haematopoietic cells when treated with appropriate differentiation inducing agents (Blagosklonny *et al*., 1996). Thus, we next determined if these events were linked to the induction of differentiation of Daudi cells by type I IFN, using the characteristic loss of surface IgM expression as a marker. (Exley *et al*., 1987a,b).

The down-regulation of surface IgM upon treatment of Daudi cells with IFNα and IFNτ occurred in a dose-response fashion (data not shown). IFNα induced a rapid down regulation of surface IgM that paralleled the induction of p21[WAF1] and was closely linked with the induction of G1 arrest in Daudi cells (Figure 3a). Similarly, IFNτ was also able to induce a rapid decrease in surface IgM expression that was closely linked to the induction of G1 arrest (Figure 3b). In contrast, IFNγ which has no effect on the expression of p21[WAF1] in Daudi cells did not affect the expression of surface IgM (data not shown). Thus, type I IFN induced differentiation of the Daudi cell line is associated with the induction of p21[WAF1] and G1 arrest. Moreover, these data are consistent with the hypothesis that the coupled c-*myc* down-regulation and p21[WAF1] induction are signals for the induction of G1 arrest and differentiation of Daudi cells following treatment with type I IFN.

To confirm that Daudi cells were indeed differentiating we examined cytospin preparations of treated and untreated cells using light microscopy. Compared to untreated cells (Figure 4a), IFNτ treated cells (Figure

4b) showed a distinct plasma cell-like morphology characterized by their large cytoplasmic volume and eccentric nuclei. With IFNα treatment, a large number of differentiated cells also appeared to have undergone changes in their nuclear structure (Figure 4c) that closely resembled cells undergoing apoptosis suggesting that the differentiation of these cells is accompanied by apoptotic cell death. As described below, the differences in morphology of IFNτ and IFNα treated cells is related to the kinetics of the apoptotic response of Daudi cells to these IFN, where higher concentrations IFNτ are required to induce apoptosis in these cells compared to IFNα over the same time period. These data suggest that type I IFN induce B cell differentiation to plasma cells and ultimately apoptotic cell death.

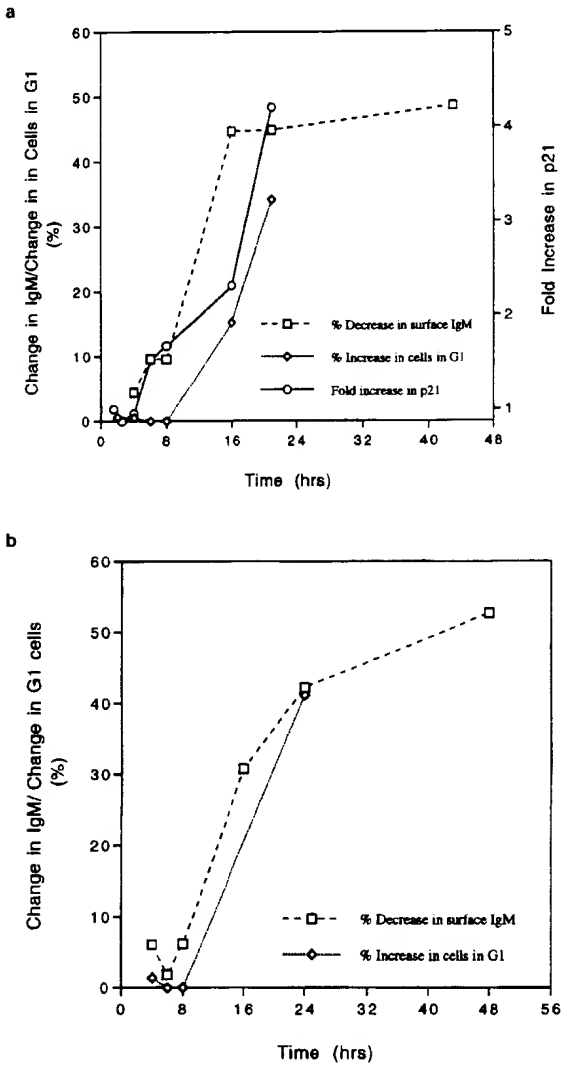

Figure 3 Relationship of G1 arrest to the differentiation response induced by IFN treatment of Daudi cells. Optimal concentrations of IFN were determined from prior dose-response experiments. Data are representative of at least two independent experiments. (**a**) Time course of the effect IFNα on surface IgM expression, p21^{WAF1} induction and G1 arrest. Cells were treated with 320 U/ml of IFNα in each case and stained for surface IgM expression or with propidium iodide for cell cycle analysis. Cell surface expression of IgM was analysed by flow cytometry of cells that were stained directly with a FITC-conjugated anti-human IgM antibody according to previously described methods (Bertolini *et al.*, 1992). A total of 1×10^6 cells were stained for each sample with an optimal concentration of antibody that was previously determined by titration. Data from 30 000 events were gathered and analysed. Percent change in G1 cells is the difference in the percent of cells with 2N DNA content between untreated and treated samples. Data for p21^{WAF1} are from densitometric scanning of the bands in Figure 1a. (**b**). Time course of the effect of IFNτ on IgM expression and G1 arrest. Cells were treated with 3200 U/ml of IFNτ and analysed as above in (**a**)

In experiments using propidum iodide staining and flow cytometry analysis of IFN treated cells, IFNα and IFNτ, but not IFNγ, were found to induce an increase in cells with a DNA content of less than 2N, suggestive of apoptotic cell death, in a dose response fashion (data not shown). Using the more specific TUNEL assay for apoptosis in conjunction with flow cytometry the results in Figure 5a confirmed that IFNα induced apoptotic cell death in Daudi cells. The time course and dose response of the TUNEL assay paralleled that of the propidium iodide staining (data not shown), and significant apoptosis was seen in the population by 3 days with 100 U/ml of IFN. The time-course of this apoptotic effect is, however, in contrast to the early strong increase in cells with 2N DNA content seen with arrest in the G1 phase of the cell cycle (see Figure 1a). Thus, apoptotic cell death occurs after the changes in the cell cycle seen in these cells. IFNτ also induced apoptosis in Daudi cells, but much higher concentrations were required to achieve the same kinetics as IFNα (Figure 5b). This is consistent with the results depicted in Figure 4 where IFNα treated cells appeared to show more pronounced apoptotic morphology than IFNτ treated cells, and support the conclusion that apoptosis follows the acquisition of the differentiated phenotype in Daudi cells. Again, IFNγ was without effect in these assays (data not shown).

In further confirmation of these results, cells treated for 3 days with IFNα and IFNτ showed significantly elevated activity of the ICE-related protease CPP32 (Figure 5c). The family of ICE-proteases that include CPP32 are recognized as one of the key end mediators of apoptosis in mammalian cells (Patel *et al.*, 1996) and are good markers for ongoing apoptosis. Furthermore, IFNτ is a weaker inducer of the activity of CPP32 than IFNα, which is consistent with the data presented above that IFNτ induces a slower apoptotic response in Daudi cells.

In this study, we have shown that type I IFN induced an ordered cell cycle arrest, differentiation and cell death response in Daudi cells that is characteristic of the terminal differentiation of B cells. In particular, the induction of G1 arrest and differentiation are closely linked to the induction of the cdk-inhibitor p21^{WAF1} by type I IFN in Daudi cells. The type I IFN induced expression of p21^{WAF1} in Daudi cells occurs within a similar time frame as the down-regulation of c-*myc* and cyclin D3, and the inhibition of pRB phosphorylation, showing that these latter changes occur in parallel or in concert with the expression of p21^{WAF1} in Daudi cells, and may be important in the IFN-induced differentiation of these cells. Finally, we showed that following differentiation, continued IFN treatment of Daudi cells induces apoptotic cell death, a feature characteristic of B cell terminal differentiation.

We have previously shown that IFNα is also able to induce the expression of p21^{WAF1} in cells of the prostate

cancer cell line DU145, which do not express a functional pRB protein, and can inhibit cdk2 activity through p21^{WAF1}-cdk2 complexes (Hobeika *et al*., 1997). The block in G1 induced by IFN in these cells is, however, leaky as the cells do progress into S phase albeit at a much slower pace than untreated cells (Hobeika *et al*., 1997). This supports the conclusion that in Daudi cells, which express a functional pRB protein, the type I IFN induced inhibition of phosphorylation of pRB probably co-operates with p21^{WAF1} induction in optimizing G1 arrest. On the other hand, the ability of IFNα to inhibit G1

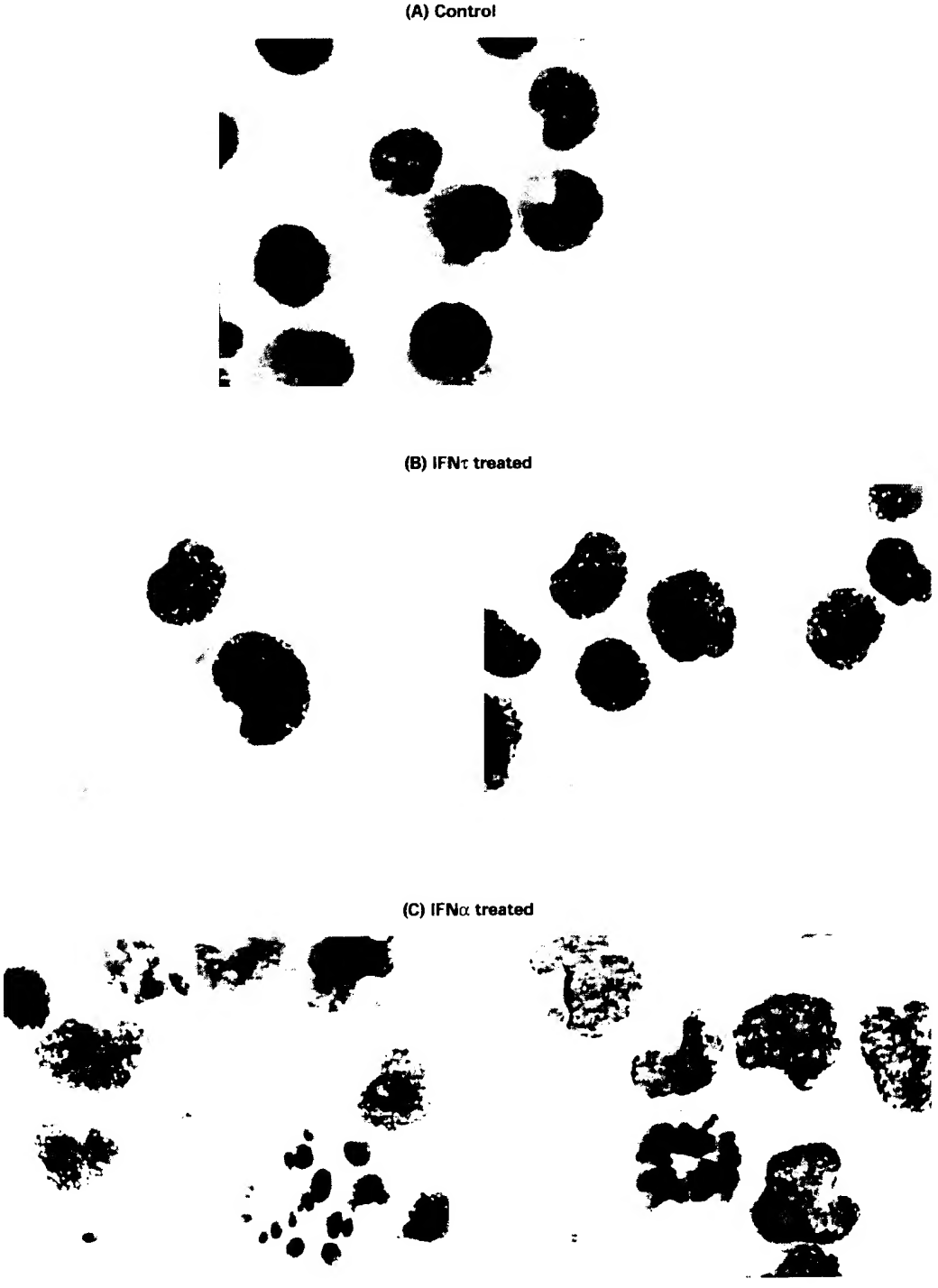

Figure 4 Morphological features of IFN treated Daudi cells (400× magnification). Cells were left untreated (control) (**a**), or treated with 3200 U/ml of IFNτ (IFNτ treated) (**b**), or 320 U/ml of IFNα (IFNα treated) (**c**) for 48 h. Cytospin preparations of cell suspensions were stained using the LeukoStat stain (Fisher Scientific, Norcross, GA) and examined by light microscopy

progression in DU145 does suggest that other p21^{WAF1}-dependent but pRB-independent mechanisms contribute to IFN-induced inhibition of growth in Daudi cells (see, for example, Dimri *et al.*, 1996).

The exact mechanisms by which type I IFN induce differentiation in Daudi cells are not known. However, the down-regulation of c-*myc* protein appears to be crucial to a successful differentiation program (Morgenbesser and DePinho, 1994), and is consistently observed in haematopoietic cells induced to differentiate by various agents (Steinman *et al.*, 1994; Blagoskonny *et al.*, 1996; Collins, 1987; Yen *et al.*, 1992). Further, in haematopoeitic cells c-*myc* down regulation appears to be tightly coupled to p21^{WAF1} induction and growth arrest (Steinman *et al.*, 1994; Blagoskonny *et al.*, 1996) and p21^{WAF1} is induced and accumulates in cells that are induced to undergo terminal differentiation by a number of differentiation inducing agents (reviewed in Gartel *et al.*, 1996). Moreover, the c-*myc* protein can antagonize the activity of p21^{WAF1} (Hermeking *et al.*, 1995), most likely through a mechanism that is analogous to its ability to interfere with the inhibitory action on cyclin-cdk complexes of another related cdk-inhibitor p27Kipl (Steiner *et al.*, 1995; Vlach *et al.*, 1996). Thus, the reciprocal relationship between the down-regulation of c-*myc* and upregulation of p21^{WAF1} shown here is most likely important to the type I

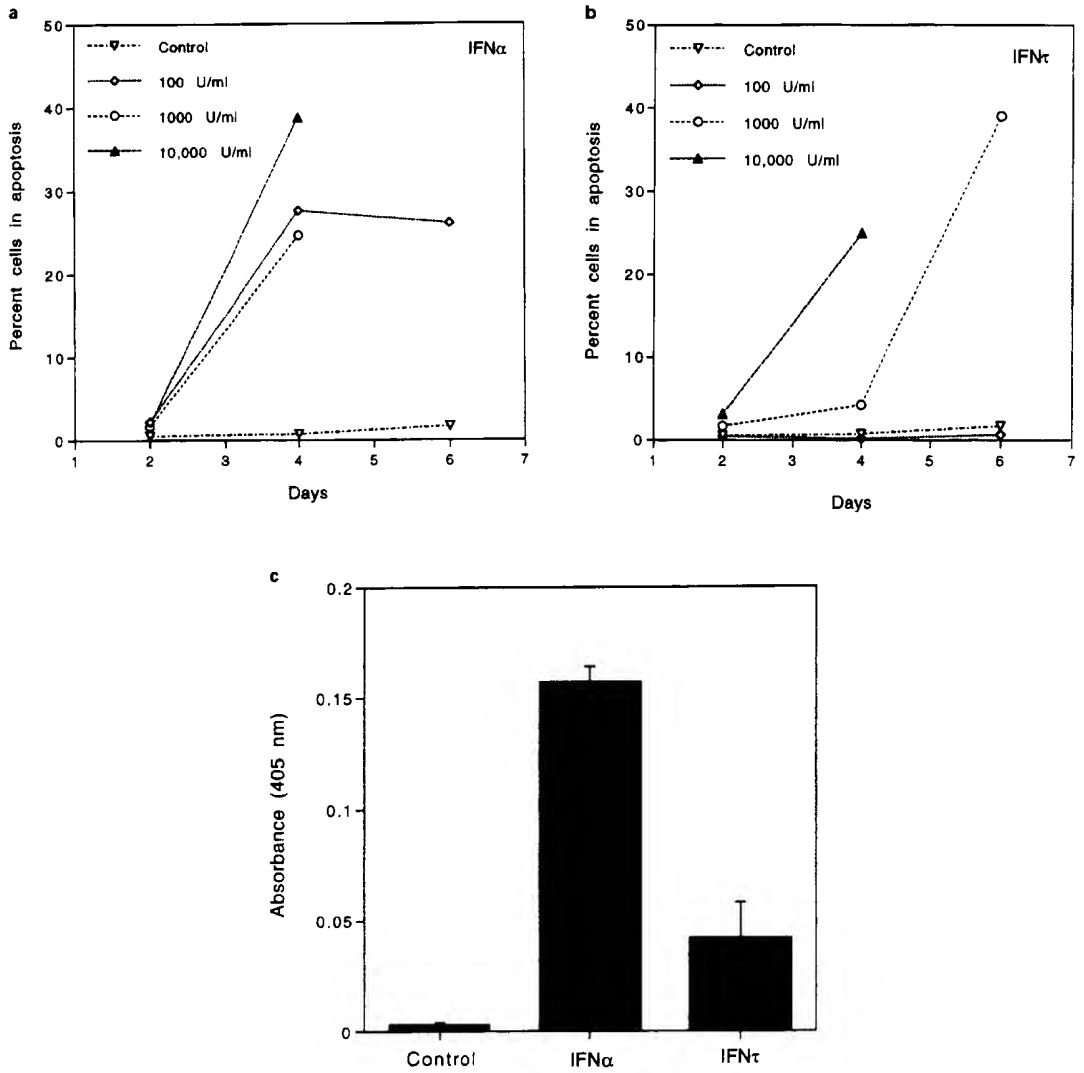

Figure 5 TUNEL assay (**a**, **b**) for DNA strand-breaks, and ICE-protease CPP32 activity (**c**), during apoptosis induced by type I IFN in Daudi cells. Data are representative of at least two independent experiments. (**a**) Cells were treated at approximately 2×10^5 cells per ml with the indicated concentrations of IFNα. Control cells were left untreated. Apoptosis was determined by the TUNEL method using the Apoptosis Detection System from Promega (Madison, WI) and flow cytometry. The percentage of apoptotic cells represents the total percentage of FITC-positive cells in G0/G1 and FITC-positive cells with a DNA content less than 2N. (**b**) Apoptotic effect of IFNτ treatment as in (**a**) on Daudi cells. (**c**) Induction of CPP32 protease activity in Daudi cells by IFN treatment. Cells were treated with 500 U/ml of IFNα (IFNα), and IFNτ (IFNτ), or left untreated (control), for 72 h. Cell lysates from these cells were assayed for CPP32 protease activity using the colorimetric ApoAlert kit from Clontech (Palo Alto, CA). For assay, cell lysates were prepared from equal number of viable cells (10^6 cells per sample). Activity was assessed by using the synthetic peptide substrate, DEVD, coupled to p-nitroanilide and following release of the chromophore at 405 nm. Specificity was assessed using the synthetic peptide inhibitor of CPP32, DEVD-CHO, supplied with the kit

IFN induced growth arrest and cellular differentiation. This ability of c-*myc* to antagonize the function of cdk-inhibitors such as p21^{WAF1} has been postulated as the reason for the proliferation and immortalization of tumor cells that occurs upon activation of c-*myc* in these cells (Hermeking *et al*., 1995). Our data show that in IFN-treated Daudi cells down-regulation of c-*myc* occurs prior to p21^{WAF1} induction, and is in fact the earliest detectable event. Since the down-regulation of c-*myc* is thought to be a differentiation signal (Steinman *et al*., 1994; Blagoskonny *et al*., 1996; Morgenbesser and DePinho, 1994; Collins, 1987; Yen *et al*., 1992), the events associated with the IFN-induced cell cycle arrest may be in response to a signal(s) for differentiation.

Acknowledgments
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Signal Transduction Pathways in Apoptosis

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Abstract. Emerging evidence indicates that apoptosis is regulated by some of the same signal transduction pathways previously implicated in other physiological cellular responses, including alterations in intracellular Ca^{2+} compartmentalization, activation of protein kinases and phosphatases, alterations in pH and oxidative stress. Interestingly, signals that promote apoptosis in one model can suppress cell death in another, indicating that cellular responses are determined by the intrinsic programming of the cell in question. This review will summarize current knowledge of the signal transduction pathways regulating apoptosis and discuss how they may be coupled to components of the molecular machinery for cell death. *Stem Cells 1996, 14 619-631*

Introduction

Apoptosis (programmed cell death) is a highly regulated process of selective cell deletion involved in development, normal cell turnover, hormone-induced tissue atrophy, cell-mediated immunity, tumor regression and a growing number of pathological disorders, typified by AIDS and Alzheimer's diseases [1, 2]. The response is characterized by a series of morphological alterations, including plasma and nuclear membrane blebbing, organelle relocalization and compaction, chromatin condensation, and the formation of membrane-enclosed structures termed "apoptotic bodies" that are extruded into the extracellular milieu [2, 3]. Uptake of this cellular debris is carefully controlled; apoptotic cells and bodies are specifically recognized and cleared by neighboring epithelial cells and professional phagocytic cells (macrophages) before their contents can be released into the extracellular milieu, thereby allowing for cell death to occur in the absence of inflammation [3].

The most familiar biochemical feature of apoptosis is endogenous endonuclease activation, resulting in the production of domain-sized large (50-300 kilobase) DNA fragments [4-6] and oligonucleosomal cleavage products commonly referred to as "DNA ladders" [7]. It appears that the smaller fragments are derived from the larger, and it has been suggested that different enzymatic activities are involved in generating each type, as evidenced by their unique cation requirements [8]. More recent work has demonstrated that a family of cysteine proteases homologous to the *Caenorhabditis elegans* (*C. elegans*) cell death gene *ced*-3 and human interleukin 1 (IL-1) converting enzyme (ICE) are also critically involved in the response, as inhibitors of these enzymes block both endonuclease activation and cell death [9-15]. The most familiar of their substrates in apoptotic cells is the 116 kD polypeptide poly (ADP-ribose) polymerase (PARP), which is cleaved at a DEVD site by one or more members of the family to yield an 85 kD fragment [16, 17]. Although ICE itself does not appear to be required for most examples of apoptosis [18], another member of the family that exhibits higher homology to *ced*-3, termed "apopain" or "CPP32," appears to be more centrally involved [13, 19, 20]. Precisely how the ICE family regulates endonuclease activation and the other features of apoptosis is not known.

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At the molecular level, a family of polypeptides homologous to *bcl*-2 appears to play particularly important roles in regulating apoptosis. One class of homologs (including *bcl*-2, *bcl*-x_L, *mcl*-1, and several viral proteins) suppresses apoptotic cell death, while another group (*bax*, *bcl*-x_s, and *bak*) promotes apoptosis sensitivity. *Korsmeyer's* laboratory has provided a possible mechanistic explanation for these differential effects on apoptosis with the observation that the BCL-2 family members form dimers which can apparently impact the process in opposite fashions: BCL-2:BAX heterodimers predominate in cells that are resistant to apoptosis, whereas a preponderance of BAX:BAX homodimers has been linked to susceptibility to cell death [21]. Most importantly, overexpression of BCL-2 or BCL-X_L blocks apoptosis induced by very diverse stimuli, including growth factor withdrawal, tumor necrosis factor (TNF), engagement of the Fas antigen, ionizing radiation, oncogenes such as *myc* and chemical chemotherapeutic agents [1], strongly suggesting that they function at a downstream site within the apoptotic pathway that is proximal to the effector machinery (proteases and nuclease[s]). Moreover, as is true for the ICE proteases, the apoptosis-regulatory functions of BCL-2 and its homologs are evolutionarily conserved, as the *C. elegans* cell death suppressor *ced*-9 is a structural and functional homolog of human *bcl*-2 [22, 23].

Regulation by Ca^{2+}

Several lines of evidence indicate that alterations in the cytosolic Ca^{2+} concentration and/or intracellular Ca^{2+} compartmentalization are involved in the regulation of apoptosis. Treatment of a variety of cell types with the endoplasmic reticular Ca^{2+} ATPase inhibitor thapsigargin or with Ca^{2+} ionophores leads to apoptosis [24-32]. Apoptosis can also be triggered in thymocytes (immature T cells) and primed mature T or B cells by antigen receptor stimulation, responses that are dependent upon sustained Ca^{2+} increases [33]. Glucocorticoid-induced apoptosis in thymocytes and certain T cell lines also involves sustained Ca^{2+} increases that are mediated via Ca^{2+} influx [34]; recent data suggest that these responses are mediated by the type 3 receptor for inositol trisphosphate, a ubiquitous, physiologically relevant Ca^{2+} channel [35]. Furthermore, stimulation of glutamate (NMDA) receptors in neurons leads to Ca^{2+}-mediated apoptosis [36], a response that may contribute to excitatory neuronal toxicity. Certain chemical toxins may also promote apoptosis by disrupting intracellular Ca^{2+} homeostasis, leading to nonphysiological Ca^{2+} increases that promote endonuclease activation and apoptotic cell death [37, 38]. Intracellular or extracellular Ca^{2+} chelators, Ca^{2+} channel blockers and calmodulin antagonists can all delay or abolish apoptosis in several model systems. In addition, overexpression of the Ca^{2+}-binding protein calbindin D-28K can block apoptotic cell death in lymphocytes [39] and prostate carcinoma cells [32] and can prevent amyotrophic lateral sclerosis (ALS) IgG-mediated cytotoxicity in motoneuron hybrid cells [40]. Finally, recent work suggests that the protective effects of the anti-apoptosis oncoprotein Bcl-2 involve alterations in Ca^{2+} compartmentalization [41-43].

In other cellular systems, however, increases in cytosolic Ca^{2+} block apoptotic cell death. For example, treatment of IL-3-dependent hematopoetic cells with calcium ionophores blocks endogenous endonuclease activation and cell death following withdrawal of IL-3 [44]. In addition, calcium ionophores block apoptosis in aged neutrophils [45], and membrane depolarization, which leads to Ca^{2+} increases in neurons, can prevent apoptosis in response to withdrawal of nerve growth factor in dependent cells [46].

The cellular targets for Ca^{2+} in initiating apoptosis are diverse. Perhaps the most obvious is the endogenous endonuclease itself, which appears to be Ca^{2+}-dependent in most (if not all) cell types [33]. Another important effect of elevated Ca^{2+} is transcriptional activation of the genes encoding the surface cell death receptor Fas and its ligand, which participate in an autocrine suicide loop in activated mature T cells. The observation that the immunosuppressants cyclosporine A and FK506 can inhibit Fas and Fas ligand upregulation and prevent cell death in some models [47] strongly suggests that the target of these immunosuppressants, the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin, is involved in these events. Finally, our more recent work has identified a Ca^{2+}-dependent nuclear serine protease as another target for Ca^{2+} [48, 49]. This protease cleaves lamin B_1 and histone H1 in

thymocytes, CLL lymphocytes, T cell hybridomas, and melanoma cell lines, and inhibitors of the protease can block endonuclease activation, suggesting that the protease directly or indirectly controls endonuclease activation.

Protein Kinase C

Much of the evidence for a role for protein kinase C (PKC) activation in apoptosis comes from studies with phorbol esters, a class of tumor promoters that act by binding to the diacylglycerol binding site on the enzyme and promoting its activation. Some studies have demonstrated that phorbol esters stimulate apoptosis in certain cell types [50-52]. It is possible that differential expression and/or activation of particular PKC isoforms is involved, as one study has shown that spontaneous apoptosis in the U-937 human myeloid line is associated with increased PKC-β and reduced PKC-ζ [53], while another group has shown that overexpression of PKC-ζ in the same cells upregulates PKC-α and PKC-β and sensitizes them to phorbol ester-induced apoptosis [51]. In addition, the selective sensitivity of a multidrug-resistant subclone of the MCF-7 human breast carcinoma to phorbol ester-induced apoptosis is associated with overexpression of PKC-α [50]. Furthermore, it has been reported that glucocorticoid-induced apoptosis in thymocytes involves selective activation and translocation of PKC-ε [54], and circumstantial evidence for a role for PKC-δ has emerged with the observation that it contains a possible ICE family cleavage site (QDN) and is proteolytically activated in apoptotic U937 cells exposed to ionizing radiation [55].

Other work suggests that PKC activation can also block apoptosis. Phorbol esters and other activators of PKC inhibit endonuclease activation in thymocytes [56], normal [57] and leukemic [58, 59] B cells, a human mammary adenocarcinoma cell line (BT-20) [60], human synovial cells [61], IL-3-dependent hematopoietic cells [62] and kidney epithelial cells [63]. Phorbol esters can also block TNF-mediated apoptosis, presumably due to their effects on the ceramide pathway to apoptosis [60, 64]. Moreover, many PKC antagonists can stimulate apoptosis [65, 66], and the protein kinase inhibitor staurosporine is being used widely as a "universal" trigger of apoptosis [67], although it has not yet been determined whether or not the actions of these inhibitors, which are notoriously nonspecific, require inhibition of PKC.

cAMP

Studies of programmed cell death within the secondary palatal epithelium during palatal fusion provided some of the first direct evidence for a role for cyclic adenosine monophosphate (cAMP) in promoting apoptosis [68]. Pharmacological cAMP agonists are also known to be cytotoxic to certain lymphoid lines in vitro [69, 70], and agents that elevate cAMP stimulate DNA fragmentation typical of apoptosis in thymocytes [71, 72]. More recent work has confirmed these observations [73, 74] and extended them to a variety of other model systems, including immortalized primary granulosa cells [75, 76], human mammary carcinoma cells [77] and various normal and transformed T and B cells [78, 79]. As one would predict, cAMP-induced apoptosis involves activation of cAMP-dependent protein kinase (PKA) [71, 80] and specific protein phosphorylation changes [81]. cAMP can also synergize to promote glucocorticoid-induced apoptosis in thymocytes and lymphoid cell lines [82, 83].

However, the effects of cAMP are definitely cell context-dependent, as good evidence has also emerged demonstrating that cAMP can block apoptosis in other model systems. Perhaps the best example can be found in neurons, where cAMP has been shown to inhibit apoptosis in response to ex vivo culture, withdrawal of nerve growth factor, or depletion of extracellular K^+ [46, 84, 85]. cAMP also prevents spontaneous apoptosis in aged neutrophils [86], in a macrophage cell line exposed to exogenous nitric oxide [87], in ovarian follicles [88] and in T cells activated via the T cell receptor [89-91]. Ongoing work is required to determine if the same molecular targets are involved in the induction and suppression of apoptosis by cAMP.

Ceramide

Some receptors stimulate sphingomyelinase and subsequently cause the hydrolysis of sphingomyelin when they bind their ligands, leading to the release of diacylglycerol and

ceramide, each of which activates its own protein kinase cascade [92]. A great deal of recent work has demonstrated that ceramide is a fairly common trigger for the apoptosis. A rapid increase in ceramide is observed in TNF-treated fibroblasts, and addition of synthetic ceramide analogs is sufficient to reproduce all of the events observed following TNF treatment [64, 65]. Similar findings have been made in cells exposed to activating anti-Fas antibodies or Fas ligand [93, 94] or ionizing radiation [95]. Furthermore, the cancer chemotherapeutic agent daunorubicin induces ceramide synthesis via a novel mechanism [96]. Evidence has also been advanced that the Reaper cell death-associated polypeptide in *Drosophila* acts via ceramide production [97], suggesting that its actions may be evolutionarily conserved. However, it is possible that in other cases ceramide acts to suppress cell death, as has been suggested concerning neurons following nerve growth factor withdrawal [98].

Ongoing efforts are aimed at identifying the downstream targets for ceramide in apoptotic cells. Ceramide is known to activate both protein kinase(s) and phosphatase(s) which may both be important for subsequent responses [92], since ceramide-activated protein phosphatase (CAPP) has been implicated in the effects of TNF on HL-60 cells [99], and the stress-activated protein kinase (SAPK)/Jun kinase (JNK) pathway has also been implicated in the effects of TNF in more recent work [100]. The idea that Jun is involved is further supported by the observation that Jun expression is induced by ceramide and that inhibition of Jun expression prevents apoptosis [101]. Activation of Ras may function upstream of these events, as Fas engagement or exposure of cells to ceramide or TNF leads to accumulation of GTP-bound Ras (the active form), and dominant negative forms of Ras prevent Fas-, ceramide- and TNF-induced apoptosis [93, 102]. Ceramide-mediated activation of Ras leads to phosphorylation and activation of Raf1 [103], which may then link Ras to SAPK/JNK and other kinase pathways. Crosstalk between the ceramide pathway and PKC is likely to determine the outcome of ceramide signaling, as phorbol esters and diglycerides are potent inhibitors of ceramide-induced apoptosis [64, 104]. A recent report indicates that the mitogenic ceramide metabolite, sphingosine-1-phosphate, prevents ceramide-mediated apoptosis by the activation of PKC [105]. The retinoblastoma gene product (Rb) may also dictate the cellular response to ceramide, in that it has been reported that ceramide induces dephosphorylation of Rb, thereby promoting its activation and resulting in cell cycle arrest at the G_1 stage [106]. This may prevent apoptosis, as we have shown that overexpression of Rb inhibits ceramide-induced DNA fragmentation in a human bladder carcinoma line (5637) [107].

Protein Tyrosine Kinases

Since virtually all surface receptors that promote survival of growth factor-dependent cells regulate cytoplasmic protein tyrosine kinases (PTKs), it could be predicted that PTKs play important roles in suppressing apoptosis. Direct evidence for this has come from the observation that protein tyrosine kinase antagonists can directly induce apoptosis [108] and can inhibit the action of many different survival factors in hematopoietic cells, including IL-2 and IL-3 [109], GM-CSF [110], and stem cell factor [111-114]. Additional examples outside the hematopoietic system include the actions of epidermal growth factor [115, 116] and basic fibroblast growth factor [115] on their target cells in tissues. Finally, a large body of evidence is now available demonstrating that the Abelson (ABL) tyrosine kinase suppresses apoptosis under a variety of circumstances, including growth factor withdrawal [117, 118], Fas engagement [119] and exposure of cells to cancer chemotherapeutics [120, 121]. The latter is likely to be of relevance to the emergence of drug resistance in chronic myelogenous leukemia, where a specific chromosomal translocation leads to fusion of the bcr and abl loci and activation of ABL protein tyrosine kinase function.

Like the other signaling pathways discussed in this review, PTKs have been implicated in promoting apoptosis. Ionizing radiation promotes PTK activation that appears required for apoptosis in B cells [122], and engagement of the Thy-1 [123, 124] or the CD4 or CD8 antigens [125] on $CD4^+CD8^+$ thymocytes promotes T cell receptor-mediated apoptosis via activation of the PTK $p56^{lck}$. Similarly, crosslinking CD19 on B cells has been reported to lead to activation of $p56^{lck}$

and to augment radiation-induced apoptosis in the cells [126].

Cyclin-Dependent Kinases

The growing appreciation of morphological and functional similarities between mitosis and apoptosis has led to an investigation into the potential involvement of the cell cycle kinases in the control of the latter. Molecular evidence for parallel control of the two responses can be found in the observation that Myc [127-129] and p53 [130-132] promote certain apoptotic responses and that both polypeptides are known to regulate cell cycle progression. The first evidence for the involvement of cell cycle-regulating protein kinases and phosphatases in apoptosis came from the observation that apoptosis induced by components of the lytic granules of cytotoxic T lymphocytes (most notably granzyme B/fragmentin) involves "premature" activation of p32/cdc2 [133], a cell cycle kinase that is regulated by cyclins A and B. Independent work by other laboratories has confirmed that a cyclin A-dependent protein kinase(s) is required for apoptosis mediated by Myc [134] and for apoptosis induced in S-phase arrested cells by staurosporine, caffeine, okadaic acid and TNF in HeLa cells [135]. Similarly, a cyclin B-dependent kinase(s) and/or cyclin E-dependent kinase(s) appear to be involved in DNA damage-induced apoptosis in HL-60 cells [136, 137], and cyclin B appears to be involved in apoptosis in PC-12 cells following nerve growth factor withdrawal [138]. Other work has implicated cyclin-dependent kinases 1 and 2 in the effects of a staurosporine analog in several different human leukemic T cell lines [139], and cyclin D1 has recently been implicated in neuronal cell death [140]. Interestingly, this last study showed that cyclin D-dependent apoptosis was blocked by Rb [140], suggesting that Rb may be a mechanism in the suppression of apoptosis. Precisely how these kinases are activated by the other biochemical signals for apoptosis remains the subject of active investigation.

Intracellular Acidification

Work from *Eastman's* laboratory was the first to clearly implicate alterations in intracellular pH in apoptosis. Working initially with Chinese hamster ovary (CHO) cells, *Eastman's* laboratory found that apoptosis induced by cancer chemotherapeutics did not clearly involve alterations in intracellular Ca^{2+} concentration, but rather intracellular acidification was identified as the trigger [141]. Further efforts identified a pH-sensitive endonuclease (DNase II) as the most likely endonuclease mediating DNA fragmentation in this system [142]. In their subsequent efforts, they showed that intracellular acidification is also linked to apoptosis in HL-60 myeloid leukemia cells exposed to the topoisomerase-II-active agent etoposide [143] and in an IL-2-dependent T cell line following withdrawal of IL-2 [144]. These results have since been confirmed and extended by other laboratories [145, 146]. Cell acidification is also associated with apoptosis induced by withdrawal of G-CSF from neutrophils [147], and an acid endonuclease has been implicated in DNA fragmentation in this cell type [148]. Apoptosis induced by Fas engagement, cycloheximide, or ultraviolet irradiation in the Jurkat T cell line also involves acidification [149]. Furthermore, inhibition of apoptosis by phorbol esters in neutrophils and HL-60 cells has been linked to intracellular alkalinization due to activation of the Na^+/H^+ antiport [62, 144-146, 150]. The latter is attractive as a general mechanism for suppression of apoptosis, as the action of colony stimulating factors in hematopoietic cells is known to involve alkalinization due to activation of the Na^+/H^+ antiport. Implicit in this model is the idea that activation of the acid endonuclease is a critical event in apoptosis in certain model systems, which would imply that there are at least two distal pathways for genome fragmentation during the response (Ca^{2+}-dependent and -independent).

Oxygen Radicals

Several lines of evidence support the involvement of reactive oxygen species ("oxidative stress") in many models of apoptotic cell death [151]. Exogenous oxidants, including redox-active quinones and peroxides, trigger apoptosis in diverse cellular systems [152-154]. In general, low concentrations of the oxidant induce apoptosis, whereas higher levels trigger necrotic cell death [153, 154]. Even in cells that are not exposed to compounds with obvious

oxidant properties, the production of reactive oxygen species and depletion of cellular protein and soluble thiols is commonly linked to apoptosis [155-157]. Furthermore, a variety of different antioxidants, including thiols (i.e., N-acetylcysteine), ascorbate, vitamin E, citrate and radical spin traps, have all been shown to block apoptosis [155, 156, 158, 159]. Finally, evidence that the apoptosis suppressors BCL-2 and BCL-X_L may inhibit apoptosis via effects on intracellular redox provides additional evidence for the importance of the mechanism [157, 160, 161]. However, it should be emphasized that hypoxia-induced cell death can involve apoptosis, arguing against an invariant role for oxygen radicals in the response [162-165]. Furthermore, some forms of oxygen toxicity do not appear to involve apoptosis, regardless of the strength of stimulus [166].

Although the mechanisms underlying oxygen radical production in apoptotic cells are still not clear, recent work has supported a role for alterations in mitochondrial function [167]. A precipitous drop in mitochondrial membrane potential ($\Delta\psi_m$) precedes the formation of superoxide radicals, exposure of phosphatidylserine on the surface of cells, DNA fragmentation, and cell death in thymocytes and other cell types undergoing apoptosis [168-172]. This drop in $\Delta\psi_m$ is prevented by inhibitors of the ICE proteases and BCL-2 [168, 172], suggesting that it may represent a committed step in the process. A role for mitochondria in apoptosis is attractive, as dysregulation of mitochondrial function has been linked to Ca^{2+} elevations and intracellular acidification [167], and a large portion of BCL-2 has been localized to mitochondria [173]. Furthermore, recent work with cell-free models of apoptosis has demonstrated roles for mitochondrial fractions [174], and in particular, the mitochrondrial protein cytochrome c [175], in promoting ICE family protease/CPP32 activation, PARP cleavage and endonuclease activation.

Conclusions and Future Directions

Although there is now good evidence implicating specific second messengers and protein kinases in the regulation of apoptosis in diverse model systems, their mechanisms of action are still obscure. Of primary importance for future work is to determine how they regulate the activation of the members of the ICE family (i.e., CPP32), and how this in turn leads to endonuclease activation and cell death. Moreover, although the apoptosis-regulatory properties of the BCL-2 family of polypeptides have been appreciated for several years, little progress has been made toward identifying their mechanisms of action; recent developments linking BCL-2 to various components of the Ras pathway may be particularly informative, as well as evidence that BCL-2 regulates Ca^{2+} compartmentalization and/or oxidative stress. Finally, it is not at all clear why signals that lead to apoptosis in one system promote cell survival in others. Presumably, this indicates that these signaling pathways lie upstream of the cell death effector machinery, and that the cellular response is dictated by the intrinsic programming of the cell. Perhaps investigating the mechanisms by which these second messengers regulate the cell cycle will also provide information on how they regulate programmed cell death.

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CUTTING EDGE

Cutting Edge: Oral Type I IFN-τ Promotes a Th2 Bias and Enhances Suppression of Autoimmune Encephalomyelitis by Oral Glatiramer Acetate[1]

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Howard M. Johnson,§ Howard L. Weiner,* and Scott S. Zamvil[3†]

IFN-τ, a novel type I IFN that possesses immunomodulatory properties, lacks toxicity normally associated with other type I IFNs. We examined the effects of oral IFN-τ alone and in combination with oral glatiramer acetate in experimental allergic encephalomyelitis (EAE). By comparison of oral administration of IFN-α, -β, and -τ to myelin basic protein-specific TCR-transgenic mice, we demonstrate these type I IFNs promote secretion of the Th2 cytokine IL-10 with similar efficiency. Whereas IFN-α and -β induced IFN-γ secretion, a Th1 cytokine, IFN-τ did not. Oral IFN-τ alone suppressed EAE. When suboptimal doses were administered orally in combination to wild-type mice, IFN-τ and glatiramer acetate had a synergistic beneficial effect in suppression of EAE. This combination was associated with TGF-β secretion and enhanced IL-10 production. Thus, IFN-τ is a potential candidate for use as a single agent or in combination therapy for multiple sclerosis. ***The Journal of Immunology*****, 2002, 169: 2231–2235.**

Interferon-β (1a and 1b) and glatiramer acetate (GA[4]; Copaxone, Cop1) are currently the only approved medications for treatment of relapsing-remitting multiple sclerosis (MS) (1, 2). These two classes of medications have distinct immunoregulatory characteristics. IFN-β exerts several effects in an Ag-nonspecific manner (1). Among its activities, IFN-β induces IL-10 secretion (3) and suppresses IFN-γ-inducible MHC class II up-regulation on APC (4). In contrast, GA, a synthetic basic random copolymer composed of tyrosine (Y), glutamate (E), alanine (A), and lysine (K), appears to preferentially affect T cells specific for CNS autoantigens (5), altering their Ag/MHC recognition not unlike altered peptide ligands (6, 7). GA also induces populations of GA-reactive Th2 regulatory cells that may provide bystander suppression in the CNS (8). Despite approval, IFN-β and GA are only partially effective MS treatments, and IFN-β, in particular, can be associated with significant side effects and potential toxicity, underscoring the importance for developing treatments that are more potent but also possess fewer potential side effects. Because currently available MS treatments alone are not entirely satisfactory, there is enthusiasm for testing medications in combination for enhanced efficacy (9, 10). In this regard, IFN-β and GA are currently being tested in combination in relapsing-remitting MS (10).

IFN-τ, a type I IFN first identified as a pregnancy recognition hormone in ruminants (11), possesses antiviral and immunoregulatory properties (11). Like IFN-β, IFN-τ induces T cell secretion of IL-10 and suppresses IFN-γ-inducible class II up-regulation on APC. Similar to other type I IFNs, IFN-τ is acid stable (11). However, in contrast to other type I IFNs, the biological activities of IFN-τ have not been associated with either significant side effects or toxicities (12, 13). IFN-τ was effective in the prevention of both acute and relapsing (11, 12) experimental allergic encephalomyelitis (EAE), a model for MS (14). IFN-τ also reversed ongoing relapsing EAE (15). In addition, IFN-τ was equally effective in EAE when given orally as well as parenterally (12, 13). Based upon these observations oral IFN-τ was tested in a phase I MS clinical trial (16). No toxicity was observed. Thus, because IFN-τ lacks toxicity and can be given orally, it is considered an attractive candidate for further evaluation in MS therapy.

In the present study, we evaluated the combination of oral IFN-τ and GA in EAE. First, we examined how oral IFN-τ alone influences T cell cytokine secretion in myelin basic protein (MBP)-specific TCR-transgenic mice. These mice, which contain a homogeneous population of naive MBP Ac1-11-specific CD4$^+$ (Th0) cells, serve as a valuable resource to test how immunomodulatory agents influence T cell activation and differentiation of CNS Ag-specific T cells. IFN-τ administration induced lymphocyte secretion of IL-4, IL-5, and IL-10 but, in contrast to IFN-α and -β, IFN-τ did not induce IFN-γ. Thus, IFN-τ supported a Th2 pattern of T cell differentiation. When administered in combination to wild-type mice at suboptimal doses, IFN-τ and GA had a synergistic beneficial effect in suppression of EAE. The combination of

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[4] Abbreviations used in this paper: GA, glatiramer acetate; MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein.

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GA and IFN-τ promoted lymphocyte secretion of TGF-β and enhanced IL-10

Materials and Methods

Mice

PL/J MBP Ac1-11-specific TCR-transgenic female mice (17) were provided by Dr. C. A. Janeway, Jr. (New Haven, CT). PL/J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Experimentation was conducted at Brigham and Women's Hospital (Boston, MA) and the University of California (San Francisco, CA) with institutional approval according to the U.S. Public Health Service's *Policy on Human Care and Use of Laboratory Animals*.

Antigens

MBP was prepared from mouse brains and purity was confirmed by gel electrophoresis and amino acid analysis. MBP Ac1-11 (Ac-ASQKRPSQRHG) was synthesized and HPLC purified.

EAE induction

EAE was induced in 10- to 12-wk-old female PL/J mice using 300 μg MBP in CFA containing 8 mg/ml H37Ra (Difco, Detroit, MI). Mice were injected on the flanks and base of the tail. A total of 400 ng of *Bordetella pertussis* toxin (List Biologicals, Campbell, CA) was administered i.v. on days 0 and 2.

Production and purification of IFN-τ

Ovine IFN-τ gene was expressed in *Pichia pastoris* using a synthetic gene construct and purified by sequential DEAE-cellulose and hydroxyapatite chromatography (12). Homogeneity was determined by SDS-PAGE and silver staining. Purified IFN-τ had a specific activity of 0.29–0.44 × 10^7 U/mg as measured by antiviral activity on Madin Darby bovine kidney cells. Murine IFN-α and IFN-β were obtained from Lee Biomolecular (San Diego, CA).

Administration of IFN-τ and GA

IFN-τ and GA were administered (100 μl each, 200 μl total volume/day) using 18-gauge feeding needles from Fisher Scientific (Norcross, GA). A total of 100 μl PBS was administered to mice treated with IFN-τ or GA alone. Mice were treated for 30 days in experiments testing EAE suppression.

T cells, proliferation, and cytokine measurements

Spleen and lymph node cells were cultured in 96-well plates at 5 × 10^5 cells/well in X-vivo 20 (BioWhittaker, Walkersville, MD) and appropriate Ag concentrations. Separate $CD4^+$ and $CD8^+$ T cells from MBP Ac1-11-

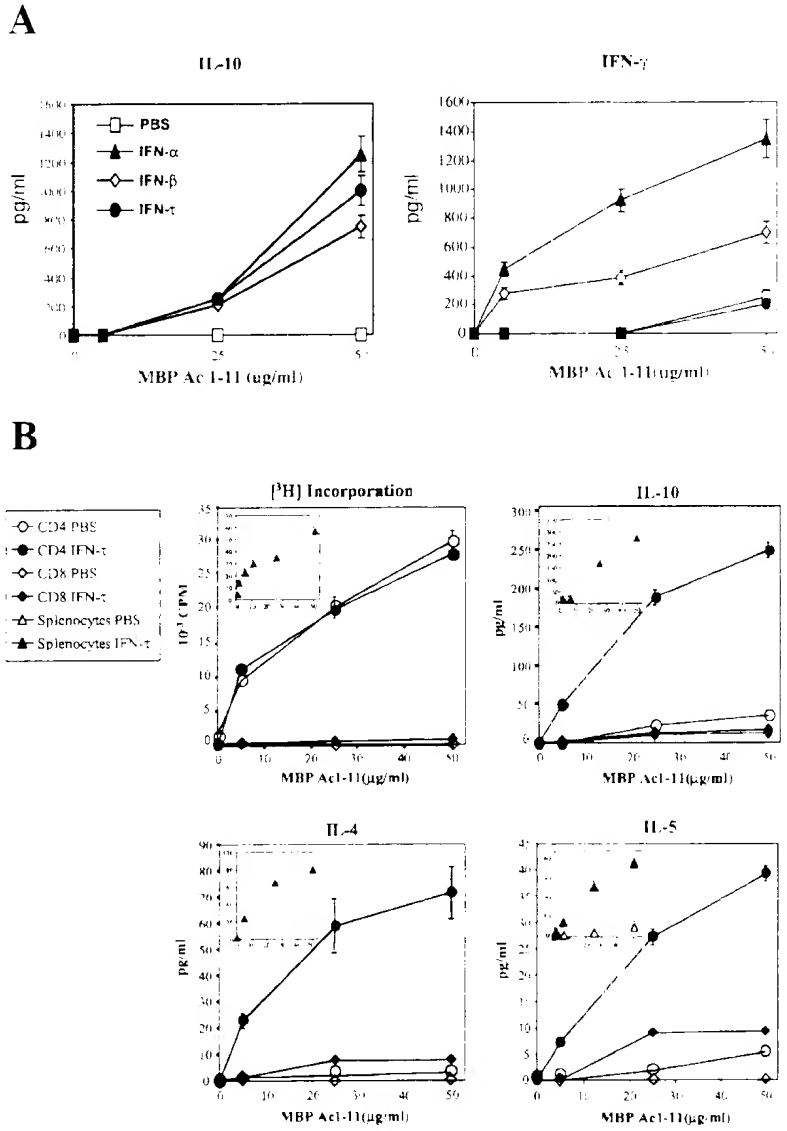

FIGURE 1. Cytokine production by T cells from MBP Ac1-11-specific TCR-transgenic mice treated with oral IFN. *A*, IFN-α, -β, and -τ induce IL-10 secretion while IFN-α and -β, but not IFN-τ, promote IFN-γ secretion. *B*, $CD4^+$ T cells from MBP Ac1-11-specific TCR-transgenic mice secrete IL-4, IL-5, and IL-10. Insets show cytokine secretion and proliferation by unseparated splenocytes from mice fed either IFN-τ or vehicle (PBS). Mice were fed daily with 1 × 10^5 U of IFN-α, IFN-β, or IFN-τ for 3 days. Splenocytes (5 × 10^5) were harvested 1 day after the last feeding. $CD4^+$ and $CD8^+$ T cells were separated as described in *Materials and Methods*. A total of 1 × 10^5 separated $CD4^+$ or $CD8^+$ T cells were cultured with 5 × 10^5 irradiated PL/J splenocytes and MBP Ac1-11. Cytokine secretion was measured by ELISA and proliferation by [^{3}H]thymidine incorporation as described in *Materials and Methods*.

specific TCR-transgenic mice were prepared by high-affinity negative selection using columns containing Ab-coated glass beads to remove B cells, monocytes, and either CD4$^+$ or CD8$^+$ cells (R&D Systems, Minneapolis, MN). CD4$^+$ and CD8$^+$ T cells were 95 and 85–90% pure, respectively, as measured by flow cytometry. For proliferation, 1×10^5 CD4$^+$ or CD8$^+$ T cells were cultured in the presence of 5×10^5 irradiated PL/J splenocytes, pulsed with 1 μCi/well [^{3}H]thymidine at 72 h, and harvested 16 h later. For cytokine measurements, culture supernatants were collected at 24 h for IL-2 measurement, 48 h for IFN-γ, IL-10, and TNF-α, 72 h for TGF-β, and 120 h for IL-4 and IL-5. Cytokines were measured by ELISA using kits from BioSource International (Camarillo, CA) as described previously (18). SE measurements for proliferation and cytokine measurements were within 10% of the mean.

Results and Discussion

The type I IFNs can induce lymphocyte secretion of the Th2 cytokine IL-10 (11). Therefore, we initially compared IFN-τ with IFN-α and -β for in vivo induction of IL-10. Unimmunized MBP Ac1-11-specific TCR-transgenic mice, used as a source of naive MBP-specific T (Th0) cells, were fed three times with 1×10^5 U of IFN-α, IFN-β, or IFN-τ. Splenocytes, isolated 1 day after the last feeding, were stimulated with MBP Ac1-11 in vitro. As shown in Fig. 1*A*, each of the three type I IFNs induced substantial IL-10 secretion. It can also be seen that IFN-τ was at least as potent as, if not more potent than, IFN-β at inducing IL-10 secretion.

It is also recognized that the type I IFNs can induce T cell secretion of the Th1 cytokine IFN-γ (11, 19). In fact, it has been observed that the frequency of IFN-γ-secreting cells increases during the first 2 mo of IFN-β 1b treatment, possibly contributing to the prominent "flu-like" symptoms that MS patients commonly experience during initial treatment (19). Thus, we compared IFN-α, -β, and -τ for in vivo induction of IFN-γ. IFN-α was approximately twice as potent as IFN-β (Fig. 1*A*). In contrast, IFN-τ did not stimulate IFN-γ secretion above the level seen in control (vehicle (PBS)-fed) mice.

While the experiments described above demonstrated that IFN-τ promoted IL-10 secretion and did not induce IFN-γ, they did not establish whether IFN-τ treatment promoted secretion of other Th2 cytokines. Thus, we examined for secretion of IL-4 and IL-5 (Fig. 1*B*). Splenocytes from IFN-τ-fed MBP Ac1-11 TCR-transgenic mice secreted IL-4, IL-5, and IL-10 when stimulated with MBP Ac1-11 (Fig. 1*B*, insets), but reduced levels of IL-2 and IFN-γ (data not shown). Similarly, lymphocytes from IFN-τ-fed wild-type PL/J mice immunized with MBP Ac1-11 or SJL/J mice immunized with encephalitogenic proteolipoprotein peptide p139–151 also secreted Th2 cytokines IL-4, IL-5, and IL-10 and reduced levels of IL-2 and IFN-γ (data not shown). To examine whether CD4$^+$ or CD8$^+$ cells were responsible for Th2 cytokine secretion, CD4$^+$ and CD8$^+$ cells were purified from IFN-τ-fed MBP Ac1-11-specific TCR-transgenic mice and restimulated in the presence of fresh APC and MBP Ac1-11. As shown in Fig. 1*B*, these Th2 cytokines were produced in cultures containing CD4$^+$ T cells. In contrast, a lower amount of IL-5 and only minimal levels of IL-4 or IL-10 were detected in cultures containing CD8$^+$ T cells. Similarly, Th2 cytokine secretion was observed in cultures containing CD4$^+$, but not CD8$^+$, T cells from IFN-τ-fed proteolipoprotein p139–151-immunized SJL/J mice (data not shown). Monocytes from IFN-β-treated MS patients can produce IL-10 (3, 20). Interestingly, the level of IL-10 production in cultures containing total spleen cells from IFN-τ-treated mice was similar to that observed when purified CD4$^+$ T cells from IFN-τ-treated mice were restimulated with fresh APC (Fig. 1*B*). While our results did not negate the possibility that monocytes may have contributed to the secretion of IL-10, they indicate that IFN-τ-induced IL-10 secretion was driven by CD4$^+$ T cells.

In a previous study, it was observed that when IFN-τ was applied in vitro at 100 or 1000 U/ml it inhibited Ag-induced proliferation by 40 or 61%, respectively (12). In contrast, while Th2 deviation was observed when IFN-τ was administered orally at 1×10^5 U and examined in vitro without additional IFN-τ, no significant inhibition of proliferation was observed in total spleen cells or in cultures containing separated CD4$^+$ or CD8$^+$ cells (Fig. 1*B*). In addition, we did not observe inhibition of proliferation in other experiments using 1×10^5 U IFN-τ or less. However, when mice were administered 1×10^6 U or higher doses in separate experiments, >50% inhibition of proliferation was observed (O. Stuve, J. M. Soos, and S. S. Zamvil, unpublished observations).

To examine how IFN-τ modulates Th1 and Th2 cytokine responses in EAE, mice were immunized for EAE induction with MBP and treated with either IFN-τ or vehicle (PBS). While MBP-specific TCR-transgenic mice are quite useful for examining regulation of Th cell differentiation (17), it can be preferable to test treatment effects on EAE induced in wild-type mice, which contain a normal T cell repertoire. Cytokine production by MBP-reactive lymphocytes was examined 20 days after immunization of PL/J

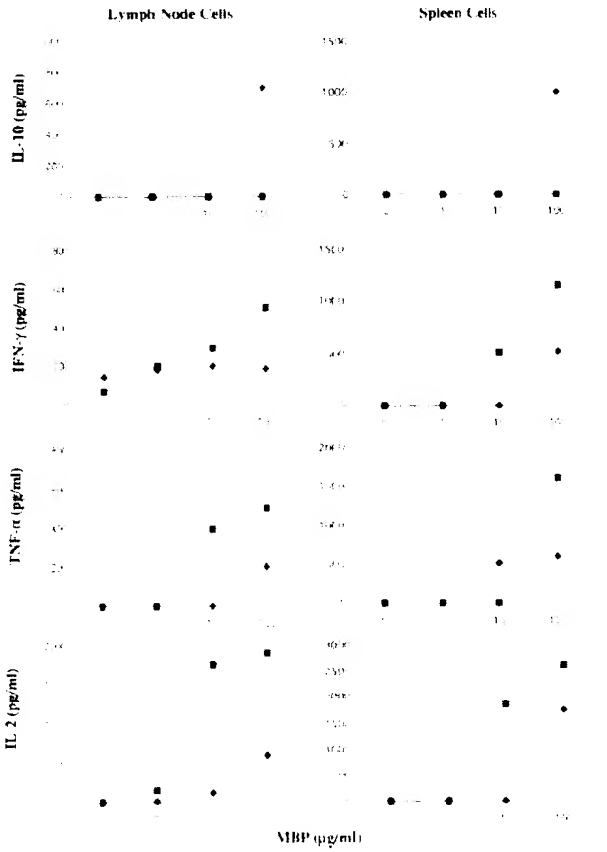

FIGURE 2. Cytokine profile of mice protected from EAE by oral IFN-τ administration. Five PL/J mice immunized with native MBP for EAE induction were fed daily for 20 days with either 1×10^5 U feeding of oral IFN-τ (◆) or PBS (■) used as a control. Mice treated with IFN-τ did not show signs of clinical EAE (EAE score of 0 for the entire duration), whereas mice fed PBS developed EAE with mean severity of 2.5. Lymph node cells and spleen cells from two mice in each group were harvested on day 21, then pooled and cultured with MBP at the concentrations indicated. One of the two mice in the PBS-fed group that was used for this analysis had a clinical EAE score of 3, while the other mouse had a score of 2. Cytokine secretion was analyzed by ELISA as described in *Materials and Methods*.

Table 1. *EAE protection by combination of oral IFN-τ and GA*[a]

Treatment	Incidence	Day of Onset	Mean Severity[b]
PBS	10/10	15.7	3.3
IFN-τ (1×10^3)	1/5	16	0.6
IFN-τ (5×10^4)	9/10	17.8	1.6
IFN-τ (10^5)	8/10	16.7	2.2
GA (100 μg)	7/10	18.5	1.6
IFN-τ (5×10^4) + GA (100 μg)	2/10[c]	18.0	0.5
IFN-τ (10^5) + GA (100 μg)	5/10	16.2	0.9

[a] PL/J mice were immunized s.c. with 300 μg MBP in CFA on day 0. A total of 400 ng pertussis toxin was administered on days 0 and 2. IFN-τ and GA were administered separately (100 μl each; 200 μl total) daily. A total of 100 μl PBS was administered to mice treated with IFN-τ or GA alone. Severity was graded as follows: 0, normal; 1, loss of tail tone; 2, mild hind limb monoparesis or paraparesis; 3, moderate paraparesis or paraplegia; 4, quadraparesis; 5, moribund/death.

[b] Mean maximal severity for each group.

[c] A value of $p < 0.001$ ($Z = 4.4$) in comparison with IFN-τ (5×10^4 U) alone; $p < 0.005$ ($Z = 2.6$) in comparison with GA alone.

mice. At this time PBS-treated mice reached an average paralysis grade of 2.5 while mice treated with IFN-τ mice did not develop EAE (see Fig. 2). Control (PBS-treated) mice that developed EAE exhibited a classic Th1 response with production of IFN-γ and TNF-α (Fig. 2). These mice also produced robust levels of IL-2 but did not produce any detectable IL-10. In contrast, MBP-stimulated lymph node cells or splenocytes from IFN-τ-treated mice secreted substantial IL-10. Lymph node cells and splenocytes from these same mice secreted less IFN-γ, TNF-α, and IL-2. Thus, IFN-τ prevention of EAE correlated with induction of IL-10 and a concomitant reduction of the Th1 cytokines, IFN-γ, TNF-α, and IL-2.

Because IFN-τ and GA have distinct modes of action (2, 8, 11) and oral administration of either one can ameliorate EAE (13, 21), we investigated whether they could complement each other when administered in combination. In preliminary experiments various doses (1×10^3, 5×10^4, and 1×10^5 U) of IFN-τ were tested alone. Whereas 1×10^5 U IFN-τ protected mice from EAE, below this amount there was a dose-dependent loss in efficacy. One hundred micrograms of GA alone was also suboptimal. However, as shown in Table 1, when IFN-τ and GA were administered in combination at suboptimal doses, mice were protected. In comparison with IFN-τ (5×10^4 U) treatment alone, there was a significant reduction ($Z = 4.4$; $p < 0.001$) in EAE incidence when mice were given combination therapy. In comparison with GA alone, combination therapy was also associated with a significant reduction ($Z = 2.6$; $p < 0.005$) in EAE incidence. These in vivo results indicated that these two immunomodulatory agents could function in an additive or synergistic manner.

GA can induce TGF-β secretion by CNS autoantigen-specific T cells (2, 21). Because previous studies attributed the beneficial affects of IFN-τ to production of IL-10 (11, 12, 15) and we observed that IFN-τ induced substantial quantities of IL-10, we evaluated whether the combination of suboptimal doses of oral IFN-τ and oral GA, which was effective in EAE protection, facilitated MBP-specific T cells to secrete TGF-β and IL-10. As shown in Fig. 3*A*, lymphocytes from MBP Ac1-11 TCR-transgenic mice treated with oral GA alone produced relatively small amounts of IL-10 when stimulated with MBP. Lymphocytes isolated from TCR-transgenic mice fed IFN-τ alone produced a moderate level of IL-10. However, mice treated with suboptimal doses of oral IFN-τ and oral GA produced approximately two times the sum of the IL-10 produced by lymphocytes from mice treated with either agent alone. In contrast to oral GA, oral IFN-τ did not induce TGF-β, and the amount of TGF-β produced by MBP-specific T cells from TCR-transgenic mice treated with the combination of oral GA and oral IFN-τ was similar to that of mice treated with GA alone (see Fig. 3*B*). Thus, the clinically beneficial effects observed when suboptimal doses of oral IFN-τ and oral GA were administered in combination (Table 1) could have reflected enhanced IL-10 secretion as well as a potential additive or synergistic effect of both IL-10 and TGF-β secretion.

It was reported that IFN-τ could either prevent or reverse relapsing EAE (12, 15). IFN-τ also prevented superantigen-induced EAE relapses (12). Furthermore, we have observed that oral IFN-τ could suppress murine collagen-induced arthritis (J. M. Soos, H. L. Weiner, and S. S. Zamvil, unpublished observation). Thus, the beneficial anti-inflammatory properties of IFN-τ are not restricted to immune responses to CNS autoantigens but may be applicable to other organ-specific autoimmune conditions. Like other type I IFNs, IFN-τ induces IL-10 when given as monotherapy (11, 12, 15). The results from previous studies (12, 15) and this investigation demonstrated that the beneficial clinical effects of IFN-τ in EAE were dose dependent. Larger in vivo doses of IFN-τ induced

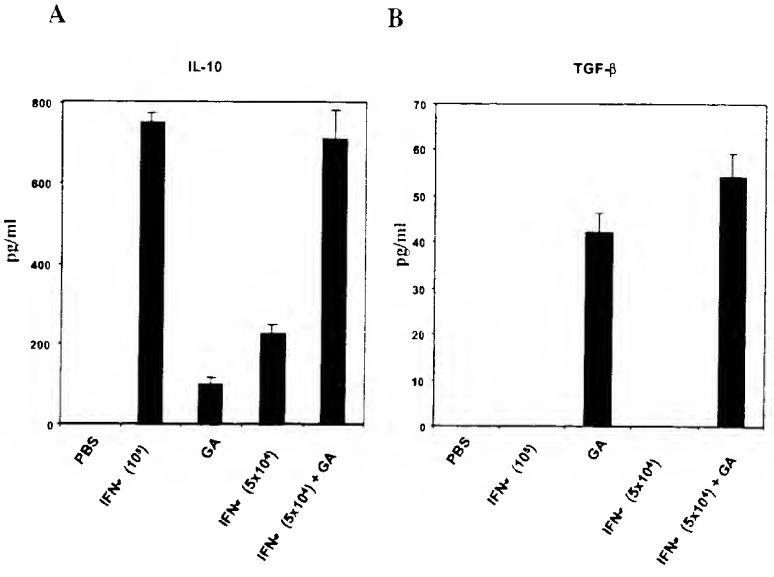

FIGURE 3. Secretion of IL-10 and TGF-β by MBP-specific TCR-transgenic mice treated with oral IFN-τ, oral GA, or a combination of oral IFN-τ and oral GA. Mice were fed daily for 3 days with PBS, IFN-τ (5×10^4 or 1×10^5 U), GA (100 μg), or the combination of IFN-τ and GA. Splenocytes and lymph node cells, harvested 1 day after the last feeding, were cultured with MBP Ac1-11 (50 μg/ml). Cytokine secretion was analyzed by ELISA as described in *Materials and Methods*.

higher levels of IL-10 by Ag-reactive T cells examined in vitro. However, IL-10 may not be solely responsible for the clinical efficacy of IFN-τ. For example, it is also known that IFN-τ suppresses IFN-γ-inducible MHC class II up-regulation (11) and, as we have shown, IFN-τ also promotes secretion of IL-4 and IL-5. Thus, it is possible that these other immunoregulatory characteristics of IFN-τ may also contribute to its beneficial effects.

Previously, it was reported that administration of oral or parenteral IFN-α in combination with GA did not improve clinical EAE (22), raising concern for the safety of using GA in combination with a type I IFN. The mechanism(s) responsible for their observation was not clearly elucidated. Interestingly, an initial clinical MS trial designed to test the safety of IFN-β 1a and GA concluded that combination was safe (10). In our study we have clearly shown that the combination of oral IFN-τ and GA is beneficial in EAE. Among other differences between the earlier EAE study and this investigation is that GA was administered parenterally in their study and not orally. Most importantly, we have also demonstrated that IFN-τ, in contrast to IFN-α or IFN-β, does not promote secretion of IFN-γ, an attractive feature of this type I IFN, which could also contribute to the different clinical observations made in these two studies. This key pharmacodynamic difference between IFN-τ and IFN-α should be considered in view of the results from a recent pilot MS trial using oral IFN-α that suggested that oral IFN-α may not be effective in treatment of relapsing-remitting MS (23). In addition, patients in that trial received either 10,000 or 30,000 U of IFN-α. In contrast, in the phase I oral IFN-τ trial (16), patients were given from 4×10^7 to 3.6×10^8 antiviral units daily, without significant toxicity.

The goal of combination therapy in MS is to improve efficacy without increasing side effects (9). Thus, medications chosen for combination therapy should not have overlapping toxicities. Theoretically, these medications should produce an additive or synergistic effect. Thus, MS medications that have different modes of action, possibly acting on different parts of the pathogenic cascade, may be preferred. In this regard, IFN-τ, like IFN-β, exerts effects in an Ag-independent manner (1), whereas GA appears to affect primarily T cells specific for CNS autoantigens (6, 8). In this report, we have demonstrated for the first time that combination of a type I IFN and GA can suppress EAE. Because oral or parenteral administration of IFN-τ is effective in EAE and is not associated with significant side effects or toxicities, IFN-τ is an excellent candidate for use as a single agent or in combination in MS.

Acknowledgments

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Stat-1 Is Not Essential for Inhibition of B Lymphopoiesis by Type I IFNs[1]

Rafael Gongora,*† Robert P. Stephan,*† Robert D. Schreiber,‡ and Max D. Cooper[2]*†§¶

Type I IFNs, IFN-α, -β, and -ω, are cytokine family members with multiple immune response roles, including the promotion of cell growth and differentiation. Conversely, the type I IFNs are potent inhibitors of IL-7-dependent growth of early B lineage progenitors, effectively aborting further B lineage differentiation at the pro-B cell stage. Type I IFNs α and β function via receptor-mediated activation of a Jak/Stat signaling pathway in which Stat-1 is functionally important, because many IFN-induced responses are abrogated in Stat-1-deficient mice. To the contrary, we show here that the inhibition of IL-7-dependent B lymphopoiesis by IFN-$\alpha\beta$ is unaffected in Stat-1-deficient mice. The present data indicate that the type I IFNs can activate an alternative signaling pathway in which neither Stat-1 nor phosphatidylinositol 3′-kinase are essential components. ***The Journal of Immunology,*** **2000, 165: 2362–2366.**

B cell development proceeds in an orderly fashion in the bone marrow (BM)[3] wherein the different differentiation stages can be characterized by the rearrangement status of the Ig genes and the expression of discriminating cell surface markers (1). In mice, the expression profiles of CD45/B220, CD43 (leukosialin), BP-1/6C3 (aminopeptidase A), and μ heavy chains allow the discrimination of pro-B cells ($B220^+CD43^+BP\text{-}1^-IgM^-$) and pre-B cells ($B220^+CD43^{+/-}BP\text{-}1^+IgM^-$) (2–4). Pre-B cells also may express a receptor formed by μ heavy chains and the surrogate light chain proteins VpreB and $\lambda 5$ (5). Productive light chain gene rearrangement occurs in postmitotic pre-B cells (6), and the subsequent expression of an IgM receptor characterizes the immature B cell ($B220^+CD43^-BP\text{-}1^+IgM^+$). Finally, coexpression of IgD begins when the newly formed B cells migrate to the periphery (7).

IL-7, an essential growth factor for B and T lymphopoiesis in mice (8), may also influence the rearrangement of B and TCR genes (9, 10). The IL-7 receptor is composed by the IL-7Rα-chain and the common γ-chain, which is shared by the IL-2, IL-4, IL-9, and IL-15 receptors (11). In keeping with the onset of IL-7 receptor expression in pro-B cells (9), B cell development is blocked at this differentiation stage in mice deficient in either IL-7 or its receptor (12, 13).

The type I IFNs, IFN-α, -β, and -ω, are members of a family of pleiotropic cytokines that participate in antiviral responses as well as in other physiological processes, such as cell growth and differentiation (14). In earlier studies, we found that the type I IFNs selectively inhibit IL-7 promoted growth of early B lineage and T lineage cells, whereas having no effect on cell growth induced by other cytokines (15, 16). The IFN-$\alpha\beta$ inhibition of IL-7-dependent cells or cell lines is featured by the induction of apoptosis. Because type I IFNs have been shown to be constitutively produced by resident BM macrophages (15), they could play a regulatory role in normal B cell development.

IFNs bind to their cell surface receptors to activate the Jak-Stat signaling system (17–19). Janus family kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) are closely related cytoplasmic tyrosine kinases that constitutively associate with the α- and β-chains of the type I IFN receptor (IFNAR). Receptor interaction with IFN-α or -β promotes Jak1 and Tyk2 phosphorylation of tyrosine residues and activates the signal activating and transcription factors Stat-1, Stat-2, and Stat-3. The Stat proteins thereby acquire the capacity to oligomerize whereupon they migrate into the nucleus and bind to regulatory motifs in the promoter regions of many genes to modulate their transcription (20). IL-7 signaling activates a partially overlapping Jak-Stat signaling pathway. Both the Jak1 and Jak3 kinases are activated after IL-7 receptor ligation, and these kinases phosphorylate multiple substrates, including the Stat-1 and the Stat-5 transcription factors (21). However, among the multiple components of the type I IFN activation pathway, Stat-1 appeared essential in that all IFN-induced responses originally examined were defective in *Stat-1*-deficient mice (22, 23). Nevertheless, this assumption can be questioned because alternative IFN signaling pathways have been identified (14, 20)

The inhibition of B and T lymphopoiesis by type I IFNs could reflect cross-talk between the type I IFN and IL-7 signaling pathways (15, 16). As a first step in examining this interaction, we examined the effects of IFN on IL-7-mediated B lymphopoiesis in *Stat-1*-deficient mice. Contrary to our expectation, *Stat-1* does not appear to be essential for the IFN inhibition of IL-7-promoted B lymphopoiesis.

Materials and Methods

Abs and immunofluorescence flow cytometry

FITC-labeled mAbs to S7/CD43, Sca-1 and IgD, PE-labeled Abs to B220 and BP-1, CyChrome-labeled Abs to CD19 and μ heavy chains, and biotin-labeled Abs to the IL-7Rα-chain were obtained from PharMingen (San Diego, CA); streptavidin (SA)-CyChrome and SA-PE were obtained from

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[3] Abbreviations used in this paper: BM, bone marrow; Jak, Janus-activated kinase; IFNAR, type I IFN receptor; SA, streptavidin; PI, phosphatidylinositol; GAF, IFN-γ activation factor; ISGF3, IFN-stimulated gene factor 3.

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Southern Biotechnology Associates (Birmingham, AL). Cell suspensions were incubated on ice for 25 min with the FITC-, PE-, CyChrome-, or biotin-conjugated mAbs, washed with PBS containing 3% FCS and 0.02% NaN_3, and counterstained with SA-CyChrome or SA-PE to reveal biotin conjugates. Before analysis with a Becton Dickinson (Mountain View, CA) FACScalibur flow cytometer, 1 μg/ml propidium iodide in PBS was added to allow identification of dead cells for exclusion from the analysis. The data was analyzed with the WinMDI 2.8 (Trotter@scripps.edu) software program.

Mice, cell lines, and IFN

Stat-1- (22) and *Ifnar1*-deficient mice (B&K Universal Group, North Humberside, U.K.) were of 129Sv/Ev background. Although the type I IFN receptor (IFNAR) is formed by two chains, IFNAR1 and IFNR2, IFN-αβ-induced responses were undetectable in $Ifnar1^{-/-}$ mice (16). *Stat-1* mice were bred as − − male × +/− female to derive +/− and −/− littermates. BALB/c mice were bred at our animal facility at the University of Alabama (Birmingham, AL). Four- to 6-wk-old animals were used as BM donors, and fetal liver cells were obtained 15 days postcoitus. *Stat-1* genotyping was conducted on DNA samples from tails or whole embryos, employing a PCR-based analysis with previously described primers (22).

The Scid7 IL-7-dependent cell line, a gift from Dr. S. I. Nishikawa (Kyoto University, Kyoto, Japan), has a pro-B phenotype ($CD19^+CD43^+BP\text{-}1^+\mu HC^-$) and is sensitive to IL-7 deprivation and the inhibitory effect of IFN-αβ (15).

An IFN-α and -β (IFN-αβ) combination isolated from viral-infected cultures and control supernatants from mock-infected cultures were purchased from Access Biomedical (San Diego, CA) and used at an equivalent concentration of 10^3 U/ml.

Ex vivo B lymphopoiesis assay

BM mononuclear cells from 6- to 8-wk-old mice or fetal liver mononuclear cells from 15-day-old embryos were cultured with IL-7-transfected NIH3T3 fibroblasts (24) at a concentration of 10^6 cells/ml in RPMI 1640 medium with 5% FCS, L-glutamine, penicillin/streptomycin, and 50 μM 2-ME in the presence or absence of IFN-αβ. Ly294002 and wortmannin inhibitors were obtained from Calbiochem (La Jolla, CA). Cultured cells were harvested by treatment with 0.02% EDTA, and viable lymphoid cells were enumerated by phase microscopy on the basis of trypan blue exclusion.

Results

Assessment of B lymphopoiesis in Stat-1*-deficient mice*

Abnormalities of B lymphopoiesis have not been noted in $Stat\text{-}1^{-/-}$ mice (22, 23), but a detailed analysis of B lineage development in these mice has not been reported. Therefore, in our initial studies, we examined B lineage cells for the expression of B220, CD19, CD43, BP-1, IgM, and IgD in BM samples from $Stat\text{-}1^{-/-}$ mice and their +/− littermate controls at 4–6 mo of age. This comparative analysis confirmed the expectation that B cell development occurs normally in *Stat-1*-deficient mice (data not shown).

Inhibition of B lymphopoiesis by type I IFNs in Stat-1-deficient mice

The possible role of Stat-1 in the IFN-αβ-mediated inhibition of B cell development was tested initially in an ex vivo system wherein progenitor cells from adult BM or fetal liver are cultivated with IL-7-transfected fibroblasts. In this ex vivo model, $B220^-$ progenitor cells undergo proliferation and progressive differentiation into IgM^+ B cells, whereas untransfected fibroblasts do not support B cell development (data not shown). Contrary to our expectation, in these experiments, the growth of $B220^+$ B lineage cells was inhibited by IFN-αβ treatment regardless of the *Stat-1* genotype. Comparable reductions in the numbers of viable $B220^+$ cells were observed in IFN-treated cultures of cells from the $Stat\text{-}1^{+-}$ and $Stat\text{-}1^{--}$ mice (Fig. 1). Phenotypic analyses over the 7-day culture interval further indicated comparable cellular composition of the surviving cell populations (Fig. 2). Notably, the development of μHC^+ pre-B and B cells was profoundly inhibited by IFN-αβ regardless of whether the progenitor cells were obtained from $Stat\text{-}1^{+/-}$ or from $Stat\text{-}1^{-/-}$ mice. The number of $B220^-$ cells was also reduced by the IFN-αβ treatment, although the limited growth of non-B lineage cells in this ex vivo system made it difficult to discern whether this was a primary or secondary effect. Titration of the IFN dosage resulted in a graduated decrease in the inhibition with lower concentrations, but differential levels of inhibition were not evident for the $Stat\text{-}1^{+/-}$ and $Stat\text{-}1^{-/-}$ cells at any IFN concentration (not shown). In keeping with previous results obtained for IL-7-dependent cell lines (15), the levels of IL-7R expression were not reduced after IFN-αβ treatment, indicating that the inhibition cannot be explained simply by deprivation of the IL-7 signal (not shown).

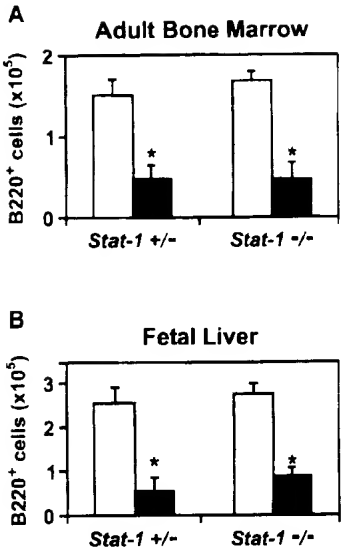

FIGURE 1. IFN-αβ inhibition of IL-7-dependent B lymphopoiesis in $Stat\text{-}1^{+/-}$ and $^{--}$ mice. *A*, Adult BM cells from $Stat\text{-}1^{+/-}$ and $Stat\text{-}1^{--}$ mice were cultured on IL-7-transfected fibroblasts for 4 days in the presence or absence of IFN-αβ. Viable cells were identified by trypan blue exclusion, counted, and the percentage of viable B lineage cells ($B220^+PI^-$) was determined by immunofluorescence flow cytometry. Results of three separate experiments are indicated. *B*, Progenitor cells in the liver of 15-day-old embryos, either $Stat\text{-}1^{+-}$ or $Stat\text{-}1^{--}$, were cultured and analyzed in the same manner as for BM cultures. Four embryos of each genotype were used in this experiment. These results were reproduced in five separate experiments. The numbers of $B220^+$ cells (±SE) recovered per 10^6 BM (*A*) or fetal liver (*B*) cells initially cultured are indicated (*, $p < 0.01$).

To confirm the specificity of the IFN-αβ inhibitory effect, we examined progenitor cells from mice deficient in the type I IFN receptor ($Ifnar1^{-/-}$). In these experiments, fetal liver cells from $Stat\text{-}1^{+/-}$ and $Ifnar1^{-/-}$ mice were cultured with the IL-7-producing fibroblasts either in the presence or absence of IFN-αβ. Whereas IFN-αβ inhibited the development of $Stat\text{-}1^{+-}$ progenitors, the development of B lineage cells from the $Ifnar1^{--}$ progenitors was unaffected by IFN-αβ treatment (Fig. 3). These results indicate that the IFN-αβ inhibitory effect is mediated specifically via the type I IFN receptor and cannot be attributed to a nonspecific toxin in the IFN-αβ preparation.

Stat-1 is essential for the IFN-αβ-mediated up-regulation of Sca-1 in early B lineage cells

The foregoing evidence indicating that Stat-1 is nonessential for the type I IFN-induced inhibition of IL-7-induced B lymphopoiesis was surprising, given that Stat-1 is essential for all of the IFN-αβ responses assessed in mature B cells (23). In view of this paradox,

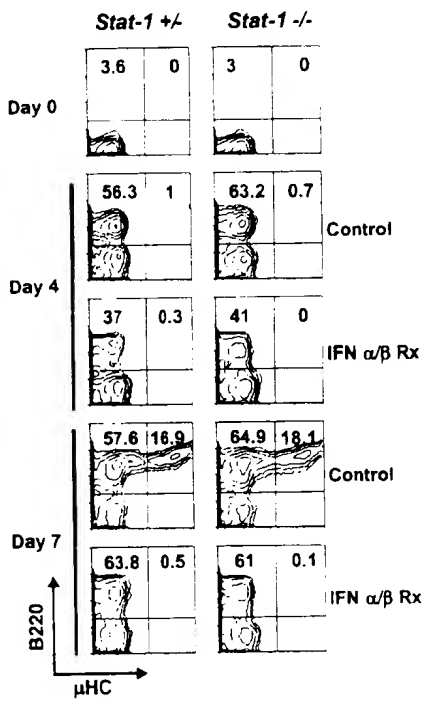

FIGURE 2. IFN-$\alpha\beta$-mediated inhibition of B lymphopoiesis in IL-7 supported cultures of fetal liver progenitors from *Stat-1*$^{+/-}$ and *Stat-1*$^{-/-}$ mice. Fetal liver cells were cultured on IL-7-transfected fibroblasts before harvesting for immunofluorescence analysis as in Fig. 1. The results of this experiment were confirmed in five separate experiments.

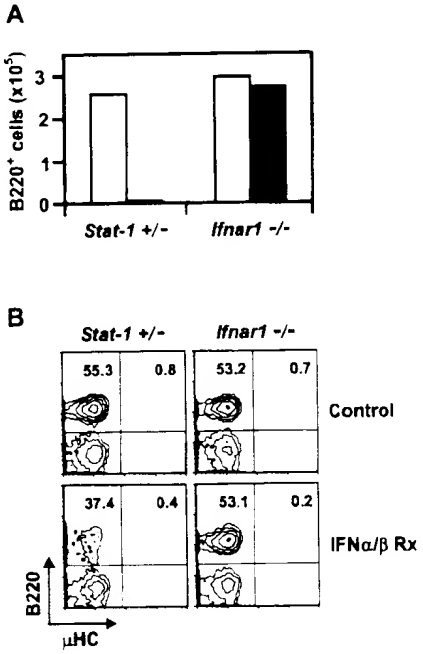

FIGURE 3. Analysis of the IFN-$\alpha\beta$ effect on B lymphopoiesis in type I receptor-deficient mice (*Ifnar1*$^{-/-}$). Fetal liver cells from *Stat-1*$^{+/-}$ and *Ifnar1*$^{-/-}$ mice were cultured for 4 days in the presence or absence of IFN-$\alpha\beta$ before enumeration and immunofluorescence analysis as in Figs. 1 and 2. The results of this experiment were reproduced in three similar experiments.

we examined whether Stat-1 was essential for another type of IFN response in the fetal liver progenitors, namely the up-regulation of transcription and expression of the Sca-1 Ag (Ly-6A/E), a cell surface accessory molecule expressed on BM and fetal liver cells (25, 26). When we examined Sca-1 expression by early B lineage cells, an IFN-induced up-regulation of this Ag could be easily detected for cells from the *Stat-1*$^{+/-}$ mice, but not for cells from the *Stat-1*$^{-/-}$ animals. IFN-$\alpha\beta$ also failed to enhance the expression of this Ag on cells from *Ifnar1*$^{-/-}$ mice, thereby confirming the IFN-$\alpha\beta$ specificity of this response (Fig. 4). Therefore, this type I IFN response is *Stat-1*-dependent in B cell progenitors as is the case for B cells

Evaluation of the role of phosphatidylinositol (PI) 3′-kinase in IFN-$\alpha\beta$-mediated inhibition of B lymphopoiesis

PI 3′-kinase, which is involved in many signaling cascades that influence cell growth, is also activated following type I IFN stimulation (27–29). To examine its possible influence in the growth inhibition induced by IFN-$\alpha\beta$, we examined the role of PI 3′-kinase in the IFN-mediated inhibition of B lymphopoiesis. The initial assessment was conducted by adding Ly294002, an inhibitor of PI 3′-kinase (30), to cultures of fetal liver cells from BALB/c mice. Although relatively high concentrations of Ly294002 could inhibit B lymphopoiesis, this PI 3′-kinase inhibitor had no demonstrable counter effect on the IFN-mediated inhibition of cell growth at any of the concentrations employed (Fig. 5*A*). The effect of this PI 3′-kinase inhibitor was also evaluated in experiments in which an IL-7-dependent pro-B cell line, Scid7, was cultured with IL-7. In these experiments, the Scid7 cells were inhibited by IFN-$\alpha\beta$ at all inhibitor concentrations tested. Ly294002 alone was again inhibitory at the highest concentrations employed, but had no demonstrable counter effect on the IFN-$\alpha\beta$-mediated inhibition (Fig. 5*B*). Wortmannin, another inhibitor of PI 3′-kinase, was also tested in these experiments with the same outcome (data not shown). The inhibitory effect of Ly294002 alone, most evident at the highest concentration employed (1 μM), can be attributed to the compromise of IL-7 signaling that involves PI 3′-kinase activity (10). If the IFN-$\alpha\beta$-mediated inhibition of cell growth also required PI 3′-kinase activity, an increase would be expected in the numbers of cells treated with both IFN-$\alpha\beta$ and the inhibitor Ly290042. This outcome was not observed at any Ly290042 concentration, thereby suggesting that integrity of the PI 3′-kinase signaling pathway is not essential for the type I IFN-mediated inhibition of B lymphopoiesis.

Discussion

The present studies indicate that Stat-1 is not essential for the IFN-$\alpha\beta$-mediated inhibition of B lymphopoiesis, in contrast with

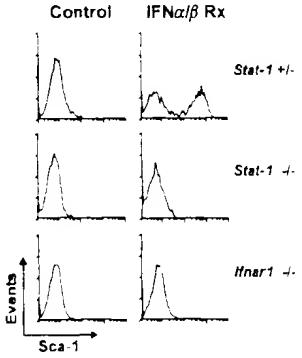

FIGURE 4. Effect of IFN-$\alpha\beta$ treatment on Sca-1 expression by B lineage cells. Fetal liver cells from *Stat-1*$^{+/-}$, *Stat-1*$^{-/-}$, and *Ifnar1*$^{-/-}$ mice were cultured for 4 days as indicated in Fig. 1 before immunofluorescence analysis of Sca-1 expression by B220$^+$ cells.

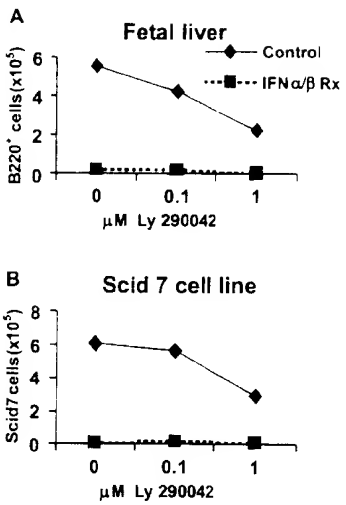

FIGURE 5. Analysis of the effect of the PI 3′-kinase inhibitor, Ly290042, on IFN-$\alpha\beta$-mediated inhibition of B lymphopoiesis. *A*, Fetal liver progenitors from E15 BALB/c embryos were cultured for 3 days with IL-7-transfected fibroblasts before treatment for 2 days with the inhibitor at different concentrations, in the presence or absence of IFN. *B*, Scid7 cells were cultured for 4 days in IL-7-containing media with or without the inhibitor and IFN-$\alpha\beta$ before enumeration and analysis as in previous figures. Cell numbers correspond to pooled cells from four separate cultures and are representative of two independent experiments.

previously described biological models wherein type I IFN-induced growth inhibition was abolished in Stat-1-deficient mice and cell lines (22, 23, 31).

Two major transcription factor complexes are formed in response to ligation of the type I IFN receptor: IFN-γ activation factor (GAF) and IFN-stimulated gene factor 3 (ISGF3) (17, 19, 20). GAF is a Stat-1 homodimer, whereas ISGF3 is formed by activated Stat-1, Stat-2, and p48 (32). Although *Stat-1*-deficient mice cannot form GAF, the possibility of a hypothetical ISGF3 complex lacking Stat-1 but still functional cannot be excluded for at least some responses. In this regard, we found that the expression of *Sca-1*, a gene containing targets for both GAF and ISGF3 factors in its promoter (26), was not up-regulated in B cell progenitors following IFN treatment of *Stat-1*-deficient mice.

The role of Stat-3 in type I IFN signaling is not as well documented as that for Stat-1 in part because the Stat-3 knockout is early embryonically lethal (33). Stat-3 may also be involved in the antiviral and antiproliferative activities of type I IFNs through up-regulation of NF-κB binding activity (34) and by functioning as an adapter to couple PI 3′-kinase to the IFN receptor, thereby activating a new signaling pathway (28, 29). PI 3′-kinase is also associated with IL-7 receptor signaling, and its activity is required for the proliferative response of B cell progenitors (10). The essential role of this signaling component is evidenced by the block in B lymphopoiesis at the pro-B cell stage in PI 3′-kinase-deficient mice (35, 36). Using inhibitors of PI-3′-kinase, Ly294002 and wortmannin, we were unable to alter the inhibitory effect of IFN-$\alpha\beta$. Nevertheless, the possibility of competition between IL-7 and IFN signaling pathways for the available pool of PI 3′-kinase was not tested in these experiments.

The indication that Stat-1 is not essential for the inhibition of B lymphopoiesis by type I IFNs suggests that another, as yet uncharacterized, transcription complex (lacking Stat-1) or a different IFN signaling pathway is responsible for this inhibition of B cell development. Interferons can inhibit the growth of embryonic fibroblasts in a Stat-1-dependent fashion involving regulation of *c-myc* expression. In this model system IFN-γ was recently shown to suppress *c-myc* expression in cells from wild-type mice, but not in those from Stat-1$^{-/-}$ mice (37). Indeed, in the Stat-1-deficient cells both *c-myc* and *c-jun* expression were rapidly up-regulated in response to IFN-γ, thereby providing evidence for Stat-1-independent signaling in cells of another differentiation pathway.

As components of the innate immune system, IFNs are produced in response to external aggression (14). They also may be constitutively produced in a normal organism. IFN-β is one of many lymphopoietic regulatory factors (reviewed in Ref. 38) produced locally in normal BM (15), where it may play a role in selection of the B cell repertoire (39). Dissociation between the IFN-induced antiviral and growth signaling pathways has also been described (40). The complexity of the IFN signaling cascades, together with the results described above, suggest an interesting versatility in the signaling components responsible for the rich diversity of IFN responses. Elucidation of the Stat-1-independent pathway that is activated in early B lineage cells via the type I IFN receptor may also reveal the mechanism whereby this signaling pathway counteracts with that of the IL-7 receptor.

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EDITORIAL

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Type I Conceptus Interferons: Maternal Recognition of Pregnancy Signals and Potential Therapeutic Agents

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Establishment of pregnancy requires that the conceptus (embryo and its associated membranes) secrete products (e.g., steroids, proteins or prostaglandins) that affect the corpus luteum (CL) directly as a luteotrophic signal (e.g., chorionic gonadotrophin), as a luteal protective signal (e.g., prostaglandin (PG)E2), or indirectly through antiluteolytic signals that inhibit uterine production of luteolytic amounts of $PGF_{2\alpha}$ to insure maintenance of corpus luteum function. Type I conceptus interferons secreted by trophoblast of sheep, cow, and goat are antiluteolytic signals responsible for maternal recognition of pregnancy that act locally on the uterine endometrium to suppress release of luteolytic amounts of $PGF_{2\alpha}$ (PGF).[1]

Type I conceptus interferons have high amino acid sequence homology with both interferon alpha II ($IFN\alpha_{II}$) and omega interferon ($IFN\Omega$).[2–4] Pig conceptuses secrete both IFNα and IFN gamma (IFNγ)[5]; however, their role in the establishment of pregnancy has not been determined.[6] Type I conceptus interferons produced by sheep, cow, and goat are called ovine trophoblast protein-1 (oTP-1), bovine trophoblast protein-1 (bTP-1) and caprine trophoblast protein-1 (cTP-1), respectively and they share considerable amino acid and nucleotide sequence homology.[1] Since the mechanisms involved in maternal recognition of pregnancy are similar for sheep, cow, and goat, this editorial will focus on oTP-1 and its role in establishment of pregnancy in sheep, as well as properties of oTP-1 that may be of therapeutic value in human and veterinary medicine.

oTP-1 is the major protein secreted by sheep conceptuses between days 12–21 of gestation that has antiluteolytic activity and the following properties[1]: (1) Mr = 19,000 kDa; (2) 3 to 4 isoelectric variants with pI values between 5.4 and 5.6; (3) nonglycosylated; (4) does not stimulate cAMP, cGMP, or inositol phospholipid-diacylglycerol second messenger systems; (5) no chorionic gonadotrophin or lactogenic biological activities; (6) potent antiviral activity; (7) high amino acid sequence homology with $IFN\alpha_{II}$ and $IFN\Omega$; (8) binds to endometrial receptors, and (9) amplifies secretion of at least 11 endometrial proteins. oTP-1 is secreted by conceptuses as early as days 8 or 10, with rapid increases (ng/uterine flushing) as conceptuses change from spherical (312 ng) to tubular (1,380 ng) to filamentous forms (4,455 ng) on day 13[7] and maximum secretion (100,000 to 150,000 ng) occurs between days 14 and 16.[8]

MATERNAL RECOGNITION OF PREGNANCY

The endometrium of cyclic ewes is stimulated by progesterone to increase phospholipid stores and cyclooxygenase enzymatic activity necessary for uterine production of luteolytic pulses of PGF, which cause structural and functional demise of the CL in cyclic ewes.[9] Actual release of luteolytic pulses of PGF, however, does not occur in cows[10] and pigs[11] until after endometrial progesterone receptors (PR) decrease, presumably due to progesterone-induced down-regulation of its own receptor[1] during late diestrus. In cows, the decrease in endometrial PR is followed by a rapid increase in numbers of endometrial receptors for oxytocin (OT-R)[10] Estrogen, acting through its receptor (ER) appears to up-regulate endometrial OT-R.[12,13] Endometrial ER and PR, depending upon stage of the menstrual cycle or estrous cycle, may be located in either epithelial or stromal cells of endometrium[14]; however, data are not available to indicate their location in sheep endometrium during the estrous cycle or pregnancy. With increased numbers of endometrial OT-R, oxytocin from the corpus luteum and or posterior pituitary stimulates endometrial release of luteolytic pulses of PGF[12] acting through the inositol phospholipid protein kinase C (IP) second messenger system.[15]

Pregnancy results in significantly lower numbers of endometrial OT-R.[15] Endometrial release of luteolytic pulses of PGF is suppressed and endometrium of pregnant ewes does not release luteolytic pulses of PGF in response to exogenous estradiol and oxytocin that do induce premature luteolysis in cyclic ewes.[1,16] However,

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the endometrium of pregnant ewes does release higher basal amounts of PGF than cyclic ewes.

Intrauterine infusion of highly purified oTP-1 alone on days 12 through 14 extends luteal lifespan in cyclic ewes from about 19 days to 28 days and inhibits endometrial secretion of PGF in response to both exogenous E and OT.[1] Recent studies with recombinant oTP-1 (roTP-1) confirmed that oTP-1 is the only antiluteolytic protein required for extension of CL function in sheep.[17,35] What is the mechanism of antiluteolytic biological activity of oTP-1? Endometrium from day 15 of the estrous cycle has high numbers of OT-R and oTP-1 receptors and has been used to demonstrate that oTP-1 does not inhibit binding of oxytocin to OT-R, oxytocin stimulation of its second messenger system, or oxytocin-induced secretion of PGF by endometrium.[1] These results suggest that initiation of secretion of oTP-1 by days 10–13 and initial intrauterine injections of oTP-1 on days 12–13 for cyclic ewes are necessary to actually inhibit synthesis of endometrial OT-R and suppress uterine release of luteolytic pulses of PGF. This is consistent with the fact that lower numbers of endometrial OT-R are detectable at this stage of early pregnancy.[15]

Intrauterine injections of 100 μg/day of highly purified oTP-1 on days 12 through 14 of the estrous cycle, i.e., beginning prior to OT-R formation, does inhibit development of endometrial OT-R as measured directly[18] or indirectly.[19] We are currently testing the hypothesis that oTP-1 either stabilize or up-regulate endometrial PR to prevent synthesis of OT-R and suppress release of luteolytic pulses of PGF. Preliminary results indicate that oTP-1 treatment of endometrium from cyclic ewes increases endometrial PR by about 40%.[1] Results from the cow, with total RNA from endometrium collected on days 10, 15, and 18 of the estrous cycle and pregnancy subjected to dot blot analysis with a cDNA probe for avian PR, indicated that steady-state levels of PR mRNA decrease between days 15 and 18 of the estrous cycle, but remain constant between days 10 and 18 of pregnancy (R.D. Geisert, C. Hostetler, F.W. Bazer, and R.C.M. Simmen, unpublished results). We hypothesize that bTP-1 is responsible for stabilization of the endometrial PR and failure of endometrium of pregnant cows to synthesize OT-R.[1] Others have determined that Type I interferons decrease receptors for estrogen and increase receptors for progesterone in tumors.[20,21] Effects of Type I trophoblast interferons on endometrial ER and PR, their location (epithelium vs stroma) and levels of ER and PR mRNAs are being determined.

Endometrial epithelial cells from day 15 cyclic ewes in culture secrete PGF in response to oxytocin; an in vitro effect inhibited by exposure of the cells to oTP-1 for 24 h prior to treatment with oxytocin.[22] A report[23] that oTP-1 and rIFNα attenuate basal production of PGF by endometrial cells cultured in vitro is not consistent with the fact that basal production of PGF is higher in pregnant than cyclic ewes[1] or with other in vivo and in vitro results.[22] The IFNα signal transduction system includes activation of phospholipase A2, which would be expected to increase arachidonic acid production[24] and synthesis of PGF. This may explain higher basal rates of secretion of PGF by endometrium of pregnant ewes and day 16 cyclic ewes exposed to oTP-1 in vitro.[1]

Temporal changes in endometrial PR during the estrous cycle and early pregnancy of sheep have not been described. However, endometrial PR and ER in cyclic ewes decrease after day 12 of the cycle and endometrial ER are lower for pregnant ewes on days 9–15.[25] Endometrial PR and ER decrease after day 13 of the bovine[10] and porcine[11] estrous cycle and, in cows, endometrial OT-R increase after the PR decline[10] as our model predicts. We hypothesize that down-regulation of PR is a prerequisite for up-regulation of OT-R and that oTP-1 and other Type I conceptus interferons stabilize or up-regulate PR to inhibit OT-R synthesis and pulsatile secretion of PGF.

High affinity, low capacity binding sites for oTP-1 are present in endometrial membranes,[26] which also competitively bind rbIFNα and rhIFNα.[27] Unoccupied oTP-1 receptors are similar for cyclic and pregnant ewes on Days 8–12, but decrease thereafter for pregnant ewes, presumably because of increased binding of endogenous oTP-1[28] or, alternatively, because of a decrease in total numbers of oTP-1 receptors. Sheep endometrium may have high and low affinity receptors for oTP-1, but only high affinity receptors for rbIFNα.[29] Antiluteolytic effects of oTP-1 may require binding to both types of receptor. If so, this would explain why oTP-1 has potent antiluteolytic activity that is not shared equally with rbIFNα and rhIFNα.[30,31] However, antiviral,[32] immunosuppressive,[33] and antiproliferative[34] properties appear to be shared equally by oTP-1, rhIFNα, and rbIFNα.

THERAPEUTIC VALUE

What is the potential therapeutic value of oTP-1? In order to answer that question it is necessary to have sufficient amounts of oTP-1 for clinical studies. To accomplish this goal a synthetic gene for oTP-1 was constructed using 11 pairs of complementary oligonucleotides containing 17 unique restriction enzyme sites spaced evenly throughout the coding region.[35] A SacII SalI fragment containing the entire open reading frame of oTP-1 was cloned in frame with a synthetic yeast ubiquitin (Ub) gene in the yeast expression plasmid pBS24Ub and named PBS24UboTP71. *S. cerevisiae* strain AB116 was then transformed with PBS24UboTP71. After a 36 h incubation of yeast strain AB116[54] containing PBS24UboTP71, approximately 100 mg soluble roTP-1 per liter of culture medium was obtained. The roTP-1 comigrated with native oTP-1 on 15% SDS polyacrylamide gels, exhibited immunological identity with natural oTP-1 using both poly- and monoclonal (HL98 2C6 3E3) antibodies to native oTP-1, and exhibited 0.6×10^8 antiviral units/mg compared to $0.7–2 \times 10^8$ antiviral units/mg for native oTP-1.[35]

The antiviral activity of oTP-1 is as potent as that of known rIFNαs, but oTP-1 does not exert the cytotoxic effects characteristic of treatment with rhIFNα and rbIFNα.[34,36] In studies of human and feline peripheral lymphocytes infected with HIV and FIV, respectively, oTP-1 exhibited no cytotoxic effects at concentrations up to 200,000 antiviral units/ml, whereas rbIFNα and rbIFNα exerted cytotoxic effects at 1,000 to 5,000 antiviral units/ml.

oTP-1 also has anticellular activity that is equivalent to or greater than that of rbIFNα and rhIFNα.[34] When anticellular activities of oTP-1, rbIFNα, and rhIFNα were compared using human amnion (WISH) and Madin-Darby bovine kidney (MDBK) cells, all inhibited progression of the cells through the S phase, but oTP-1 was more

fective at lower dosages. Also, oTP-1, at 50,000 antiviral units/ml, stopped cell proliferation without effects on cell viability, while rbIFNα, at the same concentration and antiviral activity, caused massive cell death.

Patients having steroid-dependent mammary tumors respond to treatment with IFNβ because it increases PR and decreases ER in tumor cells.[37] Our preliminary results indicate that oTP-1 also increases PR[+] and endometrial ER are lower in pregnant ewes.[25] The potential therapeutic uses of oTP-1 in treatment of patients with steroid-dependent tumors is deserving of intensive studies.

Patients suffering from infection with HIV or diseases such as hairy cell leukemia are willing to consider lifelong therapy; however, chronic IFNα treatment results in development of resistance to the effects of currently available recombinant IFNαs.[38] In addition, high doses of rIFNαs produce intolerable fever and chills, anorexia, weight loss, and fatigue[39] and they may also cause seizures.[4] Interferons have both immunoenhancing and cytotoxic effects which require that therapeutic doses be chosen that favor the immunoenhancing effects of the group. Type I trophoblast interferons act through receptors on the uterine epithelium, which is in direct contact with the trophectoderm. However, cytotoxic effects of Type I trophoblast interferons on uterine epithelium exposed to as much as 40 million units of antiviral activity per 24 h have not been detected. This led to our working hypothesis that the Type I trophoblast interferons such as oTP-1, may have unique "cell friendly" properties that will make them especially useful interferons for therapeutic purposes.

A number of diseases affecting animals result from infections by retroviruses of the family Retroviridae and the family Lentiviridae, such as the immunodeficiency viruses. These include ovine progressive pneumonia virus (OPPV), caprine arthritic-encephalitic virus (CAEV), bovine immunodeficiency virus (BIV), equine infectious anemia virus (EIAV), and feline immunodeficiency virus (FIV).[41] The diseases caused by OPPV, CAEV, and BIV, for example, are uniquely suited for testing the therapeutic value of oTP-1 in the control of immunodeficiency diseases because conceptuses of each of these species secrete Type I trophoblast interferons that have very high (80% or more) amino acid sequence homology with oTP-1. These animal models may be especially useful for assessing the therapeutic value of oTP-1 in preventing or ameliorating vertical and horizontal transmission of these viruses as well as the efficacy of oTP-1 in treating infected viruses.

SUMMARY

In summary, Type I conceptus interferons of sheep, cows and goats (perhaps all ruminants), which signal maternal recognition of pregnancy, may be useful for enhancing fertility in animal agriculture and may have uses in human and veterinary medicine as well. The Type I conceptus interferons share antiviral, anticellular, and immunosuppressive properties with Type I leukocyte interferons, but lack their cytotoxic effects, which may make them especially useful as therapeutic agents in human and veterinary medicine. The unique N-terminal structure of oTP-1 may account for its distinctive biological properties,[42] including its antiluteolytic activity.[43]

ACKNOWLEDGMENTS

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The anti-tumor activities of interferon (IFN)-α in chronic myelogenous leukaemia (CML)-derived cell lines depends on the IFN-α subtypes

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Abstract

Here we report on the anti-tumor effects of five interferon (IFN)-α subtypes, α1, α2, α5, α8, and α10 in chronic myelogenous leukaemia (CML)-derived cell lines. All of the CML cells can respond to IFN-α although the anti-tumor effects of IFN-α depend on the target cell and on the type of IFN-α subtype used. Proliferation assays showed that IFN-α8 was substantially more effective than the other four IFN-α subtypes. IFN-α8 was the most potent at upregulating immunomodulatory molecule expression while IFN-α1 was least potent. These data indicate in vitro distinctions between IFN-α subtypes that should be appreciated more in the clinic. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Interferon (IFN)-α; IFN-α8; Subtype; Chronic myelogenous leukaemia (CML); Fas

1. Introduction

Chronic myelogenous leukaemia (CML) is a cancer of the bone marrow, and is thought to arise as a direct result of the bcr-abl fusion-gene (Philadelphia chromosome positive; Ph(+)) due to translocation between chromosomes 9 and 22 [1–3]. The mechanisms favoring the growth advantage of Ph(+) cells over normal cells in CML are not fully elucidated but could be due partly to altered apoptosis and longer survival of CML clones. The BCR-ABL oncoprotein is a constitutively active protein tyrosine kinase, which plays a key role in the pathogenesis of the chronic phase of CML. It has been shown that the increased tyrosine kinase activity of BCR-ABL is a requirement for transformation and is specific to leukemia cells, and can therefore be used as a sensitive marker of the disease through molecular biology techniques. Three phases of disease activity are recognized in CML; chronic phase, accelerated phase, and blast crisis. Like many chemotherapeutic agents (e.g. members of the tyrphostin family, herbimycin A and the 2-phenylaminopyrimidine STI 571) in the CML diseases, type I interferons (IFN-α/β) have a number of therapeutic applications in the treatment of various human cancers and diseases of viral origin [3,4]. IFN-α/β play a prominent role in the control of cellular proliferation and survival as well as having potent immunomodulatory and anti-angiogenic actions. Like most human cytokines, human IFN-β is encoded by a single gene, however human IFN-α comprises a

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large family of structurally related genes expressing at least fourteen subtypes and all are on chromosome 9. The coding regions of the IFN-α genes are quite similar, and the least related are about 77% homologous [5–7]. Recent studies have clarified to some extent the biological properties of the different IFN-α subtypes and the synthesis of the different IFN-α subtypes depends on the cell type and on the IFN-α inducer used [8–10]. Despite these studies, the physiological significance of the existence of so many IFN-α subtypes remains unknown and requires addressing.

In this report, we have examined the differential responsiveness to IFN-α subtypes to six well-characterized CML-derived cell lines: Ku-812, KYO-1, MEG-O1, NALM-1, PL-21, and U-937, respectively. In particular, we have evaluated the different IFN-α subtypes for their abilities to alter cell proliferation and modulation of the level of apoptosis. We have also investigated the induction of cell surface immunomodulatory molecules, including the human leukocyte antigen (HLA) class I antigen, Fas (CD95) and FasL by the IFN-α subtypes.

2. Materials and methods

2.1. Cell lines

The CML-derived cell lines, Ku-812 [11], KYO-1 [12], MEG-O1 [13], NALM-1 [14], PL-21 [15] and U-937 [16] cell were obtained from Fujisaki Cell Center of Hayashibara Biochemical Laboratories, Inc. Each cell line was grown in a culture medium consisting of Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, and 50 mg/μl streptomycin. Cells were cultured for 24 h before the experiments to allow the cells to enter their logarithmic growth phase.

2.2. IFN-α

Recombinant human IFN-α1, 2, 5, 8, and 10 were prepared via the pET-3a – BL21 system (Novagen, WI, USA) as described previously [17]. The specific activities were 3.0×10^6 IU/mg for subtype α1, 7.2×10^7 IU/mg for α2, 4.0×10^7 IU/mg for α5, 2.9×10^8 IU/mg for α8 and 4.9×10^7 IU/mg for α10. As regards IFN-α2, because of glycosylation of the naturally occurring subtype that may influence its biologic activity, we also purified natural IFN-α2 from cultures of BALL-1 cells stimulated with Sendai virus instead of producing a recombinant product.

2.3. Growth inhibition assay

Cells of different lines were seeded at 1×10^3–5×10^3 cells/well in 96-well plates in RPMI 1640 medium supplemented with 10% FCS. IFN-α was added at the indicated concentrations (10–2×10^4 IU/ml). After 96 h, the medium was removed and the viable cells were counted using a cell-counting kit (Doujin Chemical Co., Tokyo, Japan). Triplicate wells were analyzed for each time point and IFN-α dosage. The 50% effective dose of antiproliferative effects (ED50) was estimated by Probit analysis.

2.4. Cell cycle analysis

Cell cycle analysis was carried out according to standard methods [17]. Briefly, cells of different lines were cultured at 10^4 cells/ml in RPMI 1640 medium containing 10% FCS and were left untreated or were treated with IFN-α (1000 IU/ml) for 96 h. Cells were harvested and fixed in 70% cold ethanol for 2 h. Fixed cells were stained with phosphate-buffered saline (PBS) (−) containing 40 μg/ml propidium iodide for 15 min. Cell cycle and apoptosis were analyzed with an EPICS XL flow cytometer (Beckman coulter, CA, USA).

2.5. Analysis of expression of cell-surface antigens

Cells were left untreated or treated with IFN-α (100 IU/ml) for 48 h. Next, cells were collected and approximately 2×10^4 of the cells were stained in PBS with monoclonal antibody to HLA class I molecules (#CN109; YLEM, Rome, Italy), Fas (CH-11; MBL, Tokyo, Japan) or FasL for 60 min at room temperature. The first antibody was labeled with a fluorescin isothiocianate (FITC)-conjugated anti-murine antibody for 30 min. Cells were analyzed using a Becton Dickinson FACScan Flow cytometer using standard protocols.

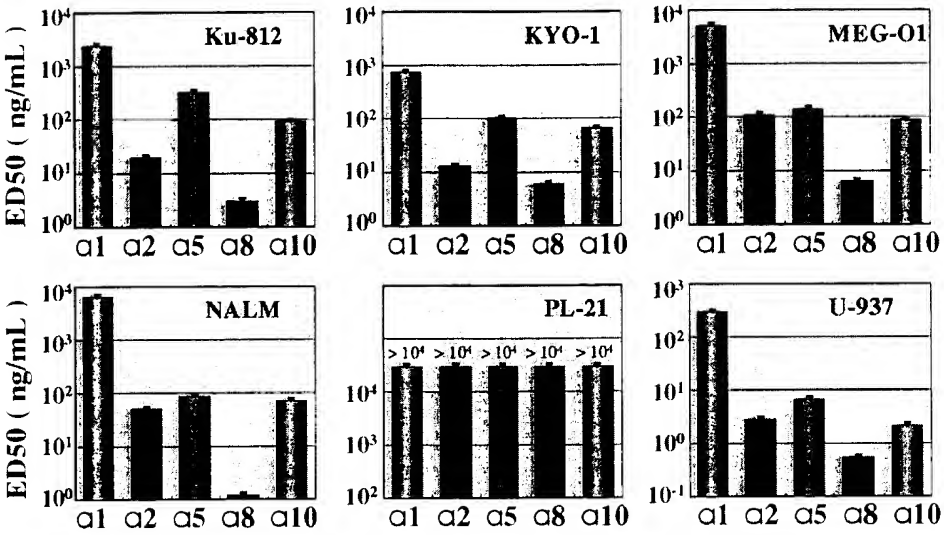

Fig. 1. Growth inhibition effects of the five IFN-α subtypes on six CML-derived cell lines. Proliferation of human CML-derived cells treated with different concentrations of various IFN-α subtypes for 96 h. The bars show the mean ED50 for IFN-α induced growth inhibition.

3. Results and discussions

3.1. Anti-proliferative effects of IFN-α subtypes on CML cell lines

The growth inhibitory effects of five IFN-α subtypes on the various CML-derived cell lines were analyzed (Fig. 1). Proliferation assays showed that sequential addition of IFN-α significantly increased growth inhibition except in PL-21 cells. The degree of sensitivity varied for each cell line. Both KYO-1 cells and U-937 cells were approximately 10-fold sensitive than the other lines in the order U-937 > KYO-1 > Ku-812 = MEG-O1 = NALM-1 ≫ PL-21. In five of the cell lines tested, IFN-α8 was substantially more effective than the other four IFN-α subtypes while IFN-α1 was the least effective. Ku-812 cells and U-937 cells, in particular, proliferated at low concentrations of IFN-α8 whereas any of the other IFN-α subtypes resulted in a reduction of cell growth by approximately 10%. IFN-α8 was at least ten times more effective than IFN-α1 in all the cell lines tested, suggesting that natural preparations with a high content of IFN-α8 would be more effective than preparations with lower levels of IFN-α8.

Interestingly, we also found a difference between the effects of the recombinant and natural IFN-α2 (Fig. 2). Anti-proliferative effects of both recombinant and natural IFN-α2 depend on the target cell lines. These differences appear to be correlated with the glycosylation of the naturally occurring subtype to some extent, however, what effects the modification of glycosylation of the mature form of native IFN-α2 have on its function are still unknown. Several groups repotted that IFN-α8 may use IFNAR1 in a different fashion and make use of separate signal transduction pathways [18,19]. Recent studies revealed that IFNAR messenger RNA expression correlated with the response to IFN therapy and therefore, the dependency of IFN-α efficacy on the target cell and on the constituent IFN-α subtypes may be explained by differences in cell surface IFNAR expression on the different target cell lines used [20].

3.2. Measurement of IFN-α induced apoptosis of CML cells

To analyze IFN-α induced apoptosis in CML-derived cell lines, we next analyzed for changes in the cell cycle by measuring the cellular DNA contents (Fig. 3). When KYO-1, NALM-1 and U-937 cell were cultured for 96 h with IFN-α (10–1000 IU/ml), the frequency of the cells showing characteristic features

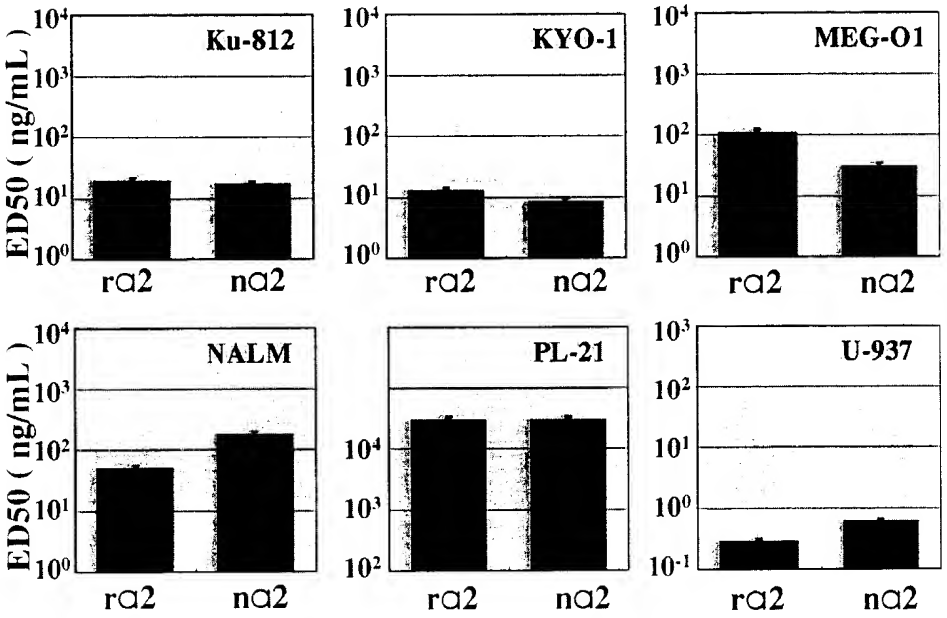

Fig. 2. Anti-proliferative effects of recombinant and natural IFN-α2. Proliferation of human CML-derived cells treated with different concentrations of both recombinant IFN-α2 (rIFN-α2) and natural IFN-α2 (nIFN-α2) for 96 h. The bars show the mean ED50 for IFN-α induced growth inhibition.

of apoptosis tended to increase with IFN-α concentration. On the other hand, Ku-812, MEG-O1, and PL-21 cells were little affected by any of the IFN-α subtypes. Concerning growth inhibition activities, IFN-α8 was 2–10-fold more potent than IFN-α1, α2, α5, and α10. However, this response appears to be unrelated to cell apoptosis.

Fas, one of the death-associated cell surface proteins induced by IFN-α, can be triggered by an appropriate death-promoting ligand (FasL) to activate downstream caspases pivotal in initiation of programmed cell death [21,22]. We examined whether there were any changes in the cellular expression of Fas to reveal first insights into the changes in the mechanisms of IFN-α-induced apoptosis in vitro. KYO-1 cells and U-937 cells showed strong IFN-α-

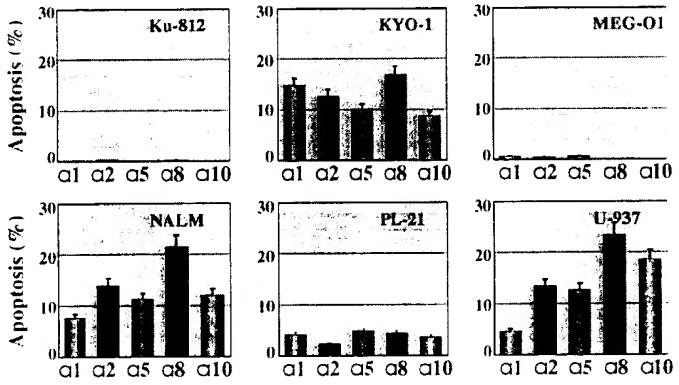

Fig. 3. Analysis of IFN-α-induced apoptosis when treated with 1000 IU/ml IFN-α for 96 h. IFN-α induced apoptosis and cell cycle distribution was determined from the intensity of PI fluorescence.

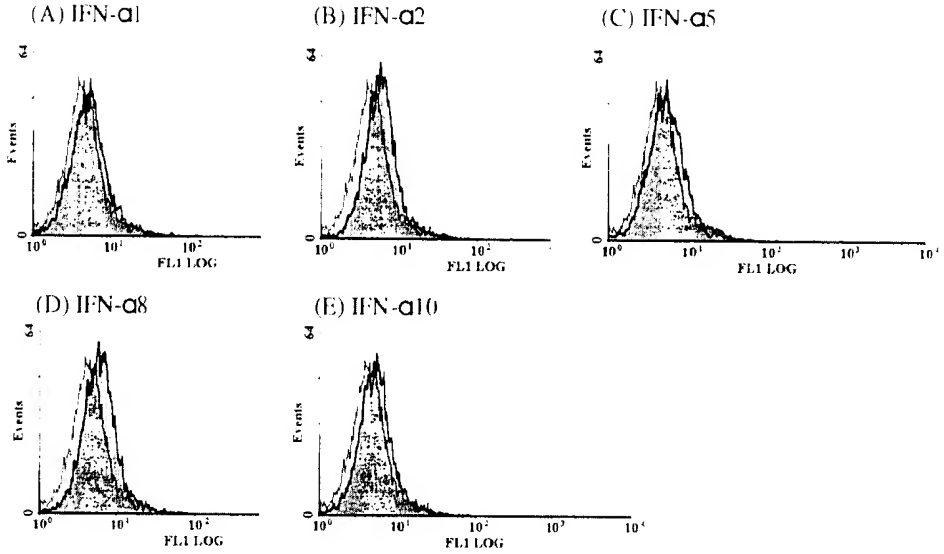

Fig. 4. Induction of cell surface Fas antigen expression on U-937 cells treated with 100 IU/ml of the various IFN-α subtypes for 48 h. Shaded and white histograms represent KPK-13 cells treated with IFN-α and untreated cells, respectively. (A) IFN-α1; (B) IFN-α2; (C) IFN-α5; (D) IFN-α8; and (E) IFN-α10

induced expression of Fas (Fig. 4). In particular, IFN-α8 was the most effective immunomodulator and IFN-α1 was the least potent. In addition, NALM and MEG-01 cells showed intermediate induction of Fas while Ku-812 and PL-21 cells were little affected by IFN-α. We also investigated the up-regu-

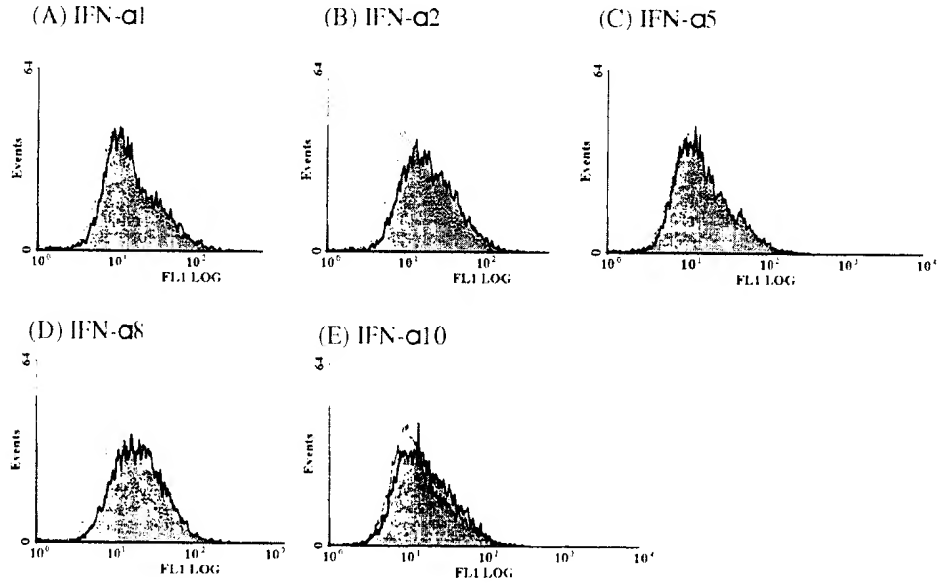

Fig. 5. Induction of cell surface HLA class I antigen expression on KYO-1 cells treated with 100 IU/ml of the various IFN-α subtypes for 48 h. Shaded and white histograms represent KYO-1 cells treated with IFN-α and untreated cells, respectively. (A) IFN-α1; (B) IFN-α2; (C) IFN-α5; (D) IFN-α8; and (E) IFN-α10.

lation of FasL. However, only U-937 cells showed IFN-α8-specific up-regulation of FasL, and IFN-α stimulation had a little effect on the expression levels when compared with unstimulated cells (data not shown). The differences in apoptosis induction could be correlated with the variations in death associated molecule expression of each cell line. Our results suggest that the Fas system may be one of the more important mechanisms of IFN-α specific induction of apoptosis in CML derived cell lines. The mechanisms underlying the cellular proliferative and apoptotic pathways involved are complex, with probable involvement of multiple protein networks/cascades.

3.3. IFN-α-induced upregulation of HLA class I expression

To examine the IFN-α induced expression of immunomodulatory molecules such as HLA class I on the CML cell surfaces, FACS analysis was employed. FACS analysis showed that IFN-α induced expression of the HLA class I in all of the six cells tested including PL-21 cells that are little affected as regards anti-proliferative effects, by any of the IFN-α subtypes. The degree of HLA class I upregulation varied for each cell line. In particular, KYO-1 cells and U-937 cells showed marked IFN-α induced enhanced expression of HLA class I molecules. The intensity of HLA class I expression was reflected by shifts in the mean fluorescence intensity as determined by the cytometer. On the IFN-α subtype level, IFN-α2 and IFN-α8 were the most effective immunomodulators (Fig. 5). Essentially the same results were obtained in studies using IFN-α subtypes on the other cell lines. Considerable attention has been focused on the ability of IFN-α to modulate cell surface antigen expression on a variety of human cells [23,24]. The ability of tumor cells to present tumor-associated antigens on the cell surface via HLA class I molecules is necessary for the generation of an effective specific anti-tumor antigen-directed CTL response. Because immunotherapy may be one of the most promising approaches for treating CML, understanding the mechanisms by which these tumors circumvent cytokine signaling, thereby evading anti-tumor specific antigen immunity, would greatly improve the efficacy of such therapy.

In summary, we have examined and estimated the in vitro anti-tumor activities of five IFN-α subtypes on six CML cell lines. We found that in all of the six cell lines tested, IFN-α8 was the most potent anti-tumor IFN-α subtype. Our results also imply that natural IFN-α preparations with high proportions of IFN-α8 would be very effective anti-tumor agents. The importance of the existence of several IFN-α subtypes has yet to be fully appreciated, but uncertainties exist in many areas including the mechanism for the subtype-specific signaling pathways and different biological activities. Further study on the significance of IFN-α subtype function will be our future objective. In particular, IFN-α8 can contribute to the further analysis of the physiological roles of IFN-α subtype-specific effects.

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